



Mechanisms Behind the Anti-Diabetic Effects of Caffeine in a Type 2 Diabetes Model of Rats

by

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As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, under the School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, from February 2017 to December 2018, under the supervision of Prof. Shahidul Islam.

This study is an original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1:

Rebecca Reddy, Ochuko Lucky Erukainure, Md. Shahidul Islam (2018) The effects of caffeine on oxidative stress, small intestinal glucose absorption, muscle glucose uptake and inhibition of key enzymes linked to type 2 diabetes (**Under preparation**)

Publication 2:

Rebecca Reddy and Md. Shahidul Islam (2018) Mechanisms behind the anti-diabetic effects of caffeine in a type 2 diabetes model of rats (**Under preparation**)

Other Publication:

Rebecca Reddy, Chika Ifeanyi Chukwuma, Ramgopal Mopuri, Ochuko Lucky Erukainure, Neil Anthony Koorbanally, Md. Shahidul Islam (2018) Antioxidative, Antidiabetic and Antiobesogenic Potentials of White, Green and Black Teas: A Comparative Study (**Under preparation**)

LIST OF PRESENTATIONS IN RESEARCH DAYS AND CONFERENCES

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3. **Rebecca Reddy**, Chika Ifeanyi Chukwuma, Ramgopal Mopuri, Ochuko Lucky Erukainure, Neil Anthony Koorbanally, Md. Shahidul Islam (2018): Antioxidative, Antidiabetic and Antiobesogenic Potentials of White, Green and Black Teas: A Comparative Study. Presented at the College of Agriculture, Engineering and Science, Research Day, University of Kwazulu-Natal, Durban, South Africa, 28 October 2017.
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LIST OF ABBREVIATIONS

ABBREVIATION	MEANING
4-HNE	4- hydroxynonenal
AA	α - amylase
AG	α - glucosidase
cAMP-PDE	adenosine monophosphate phosphodiesterase
CHO	Carbohydrate
DM	Diabetes Mellitus
EE	Energy expenditure
FFA	Free fatty acids
GLUT4	Glucose transporter type 4
GPx	Glutathione peroxidase
IDF	International Diabetes Federation
IGF-1	insulin-like growth factor 1
LDL	Low density lipoprotein
MDA	Malondialdehyde
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SGLT1	sodium-dependent glucose transporter
SOD	Superoxide dismutase
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Trx	Thioredoxins

ABSTRACT

Caffeine has been used for many years and is one of the most extensively consumed active food ingredient throughout the world. Caffeine has been broadly studied in a variety of areas regarding human health and performance. Various reports have shown that consumption of coffee or caffeine containing drinks are associated with the reduction of type 2 diabetes related symptoms, promotes weight loss and acts as an antioxidant. However, the fundamental mechanisms have not been understood. Therefore, the main objective of this study was to investigate the mechanisms behind the anti-diabetic effects of caffeine. Various *in vitro*, *ex vivo* and *in vivo* models were used to achieve this objective. The results of this study showed that caffeine possessed strong antioxidant potential and was able to inhibit key enzymes linked to type 2 diabetes *in vitro*. The results of this study further demonstrate that caffeine can modulate T2D-induced oxidative stress in various organs *in vivo*. Caffeine was able to reduce small intestinal glucose absorption, increase muscle glucose uptake *ex vivo*, improve pancreatic β -cell function and stimulate insulin secretions in an animal model of type 2 diabetes. Pancreatic histopathology showed that caffeine ameliorated T2D-induced pancreatic β -cell destruction and their functions at the end of the study. Data of this study suggest that caffeine can be used an anti-diabetic supplement in anti-diabetic foods and food products, however, a safer effective dose still needs to be identified. Hence, further studies are warranted in experimental animals and humans to determine the most effective and safer dose of caffeine for achieving its maximum anti-diabetic effects.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Diabetes mellitus (DM) is a chronic condition that occurs when there are raised levels of glucose in the blood because the body cannot produce any or enough insulin or use insulin effectively (DeFronzo *et al.*, 2015). Insulin is a hormone produced in the pancreatic beta-cells of the body, and it is responsible for transporting glucose from the bloodstream into the body's cells where the glucose is converted into energy. The deficiency of insulin or the incapability of the cells to react to insulin results in high levels of blood glucose. Hyperglycemia can cause impairment to several body organs, resulting in the development of various health complications such as cardiovascular disease, neuropathy, nephropathy and eye disease, leading to retinopathy and blindness.

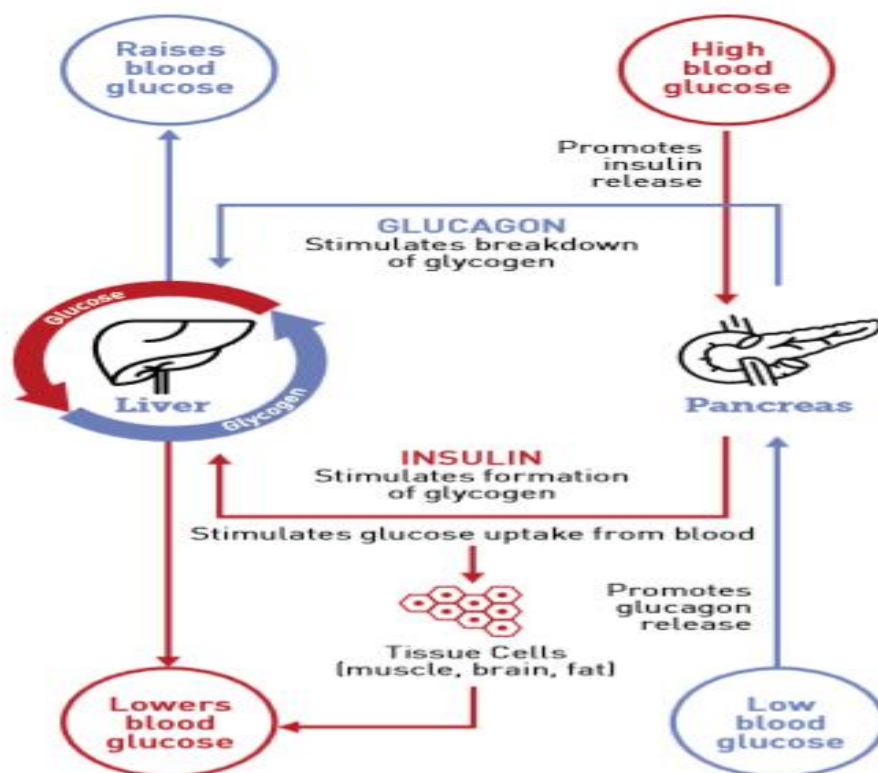


Figure 1.1: Roles of insulin and glucagon in blood glucose homeostasis (IDF Diabetes Atlas, 2017)

1.1. Prevalence of diabetes

According to International Diabetes Federation (IDF), the incidence of diabetes estimated for the years 2017 and 2045 are 87% to 91%. Approximately 425 million people worldwide are estimated to have diabetes of which 79% live in the low and middle-income countries. IDF projected that by 2045, 693 million people will have diabetes if this middle income continues.

Globally, 727 billion USD are being spent annually by individuals with diabetes as their healthcare expenditure.

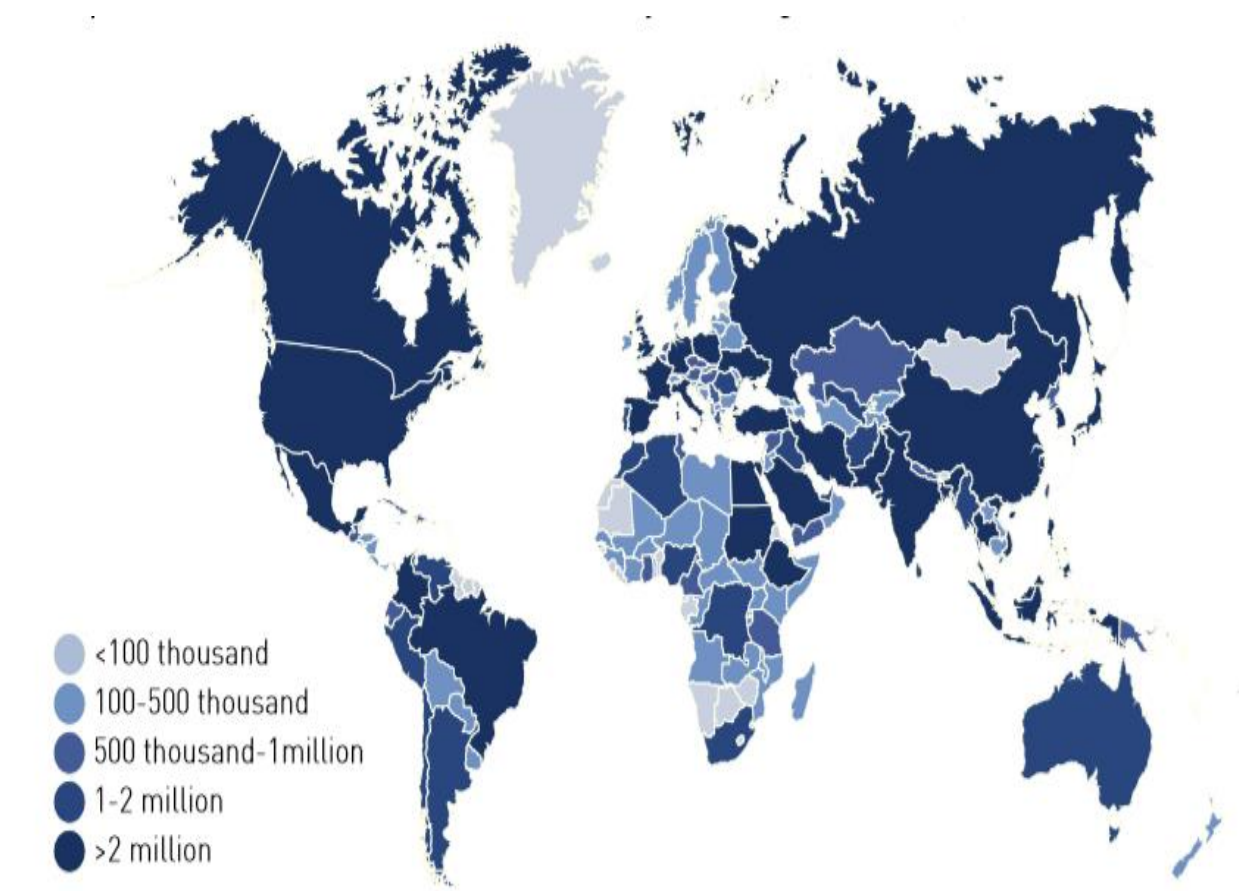


Figure 1.2: Estimated total number of adults (20-79 years) living with diabetes, 2017 (IDF Diabetes Atlas, 2017)

Table 1.1: People living with diabetes (20-79 years) who are undiagnosed in the different regions of the world (IDF Diabetes Atlas, 2017).

Rank	IDF Region	Proportion undiagnosed	Number of people with undiagnosed diabetes
1	Africa	69.2%	10.7 million
2	South-East Asia	57.6%	47.2 million
3	Western Pacific	54.1%	85.9 million
4	Middle East and North Africa	49.0%	19.0 million
5	South and Central America	40.0%	10.4 million
6	Europe	37.9%	22.0 million
7	North America and Caribbean	37.6%	17.3 million

It has been estimated that globally as many as 212.4 million people with diabetes are not aware of their illness. Since half of the people with diabetes are undiagnosed, it is essential to monitor, diagnose and offer suitable care to subjects with diabetes. People with undiagnosed diabetes are subject to higher usage of healthcare services in comparison to people without diabetes, and therefore more likely to experience higher healthcare expenditures (IDF Diabetes Atlas, 2017).

1.1.1. Type 1 diabetes

Type 1 diabetes (T1D) is triggered when the body's immune system attacks the insulin-producing beta cells in the islets of the pancreas gland. As a result, the body produces little or no insulin. Various factors such as genetic predisposition and environmental factors such as viral infection, toxins or some dietary factors have been associated with type 1 diabetes (Hinneburg *et al.*, 2006). The disease can progress at any age, but type 1 diabetes transpires more commonly in children and adolescents. People with type 1 diabetes require regular insulin injections to maintain the glucose level at a physiologically safer range. However, the prevalence of type 1 diabetes is significantly lower than type 2 diabetes.

1.1.2. Type 2 diabetes

Type 2 diabetes (T2D) is the most common type of diabetes, accounting for around 90% of all cases of diabetes (Holman *et al.*, 2015). In type 2 diabetes, hyperglycemia is the consequence of insufficient production of insulin and the inability of the body to respond fully to insulin. Factors such as consumption of unhealthy diet, high simple carbohydrate and high fat diet, obesity, physical inactivity, increasing age as well as ethnicity and family history are considered as the major causal factors for the development of type 2 diabetes. Common risk elements mentioned above give rise to an oxidative stress setting, which could modify insulin sensitivity either by increasing insulin resistance or by impairing glucose tolerance (Rains and Jain, 2011). Oxidative stress is one of the major causal factors for type 2 diabetes.

1.2. Oxidative stress

Oxidative stress can be defined as a state of imbalance between free radicals (superoxide or hydroxyl radicals) and antioxidants like superoxide dismutase (SOD), catalases and glutathione peroxidases (GP_X) and so on (Styskal *et al.*, 2012). Under oxidative stress condition, free radicals that are not reduced or removed from the cellular environment can cause damage to nucleic acids, lipids and proteins (Mathews *et al.*, 2000). Factors such as obesity, hyperglycemia and hyperlipidemia have been shown to promote oxidative stress through

elevated reactive oxygen species (ROS) production or reduced antioxidant defense (Mathews *et al.*, 2000). Diabetic subjects show increase in ROS generation. Free radicals are uncharged molecules having unpaired electrons which react with biomolecules owing to decreased enzymatic anti-oxidants activity. Antioxidants can react with free radicals by donating an electron, thereby stabilizing it. Antioxidant components have attracted great attention because of their capability to scavenge free radicals by inhibiting oxidation (Perez *et al.*, 2009).

Most living organisms possess proficient enzymatic and non-enzymatic defense systems against excess production of ROS and reactive nitrogen species (RNS). However, different external factors decrease the capability of such endogenous antioxidant defenses, resulting in disturbances of the redox equilibrium that is established in healthy conditions. Increased levels of free radicals result in impairment of cellular proteins, membrane lipids and nucleic acids, which will ultimately result in death of the cells in the body (King and Loeken, 2004). Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Maritim *et al.*, 2002).

1.2.1. Oxidative stress and the antioxidant defense system

Oxidative stress is defined as a disproportion of elements that produce ROS such as superoxide or hydroxyl radicals and the enzymes that defend the cells from these pro-oxidants are superoxide dismutases, catalase, and glutathione peroxidases (Pérez-Matute *et al.*, 2009).

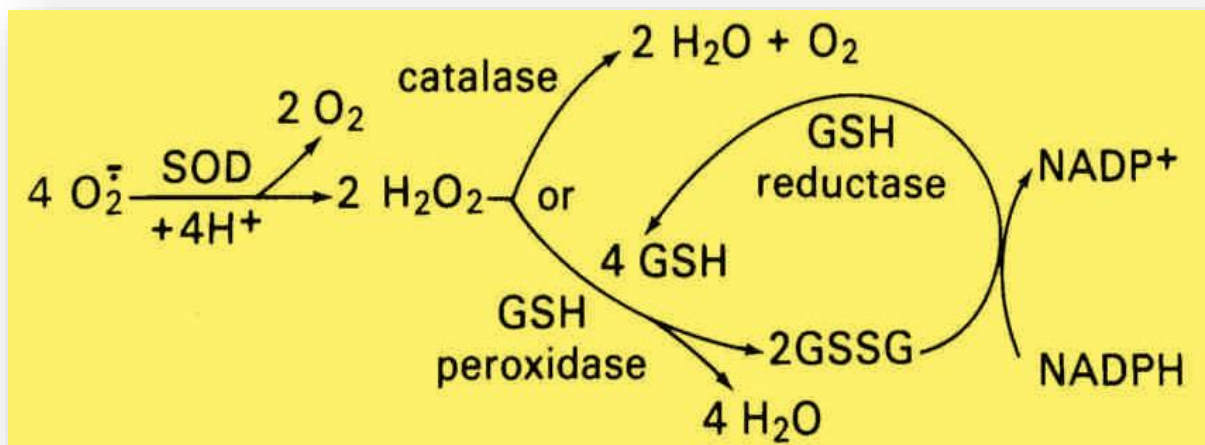


Figure 1.3: Schematic diagram of pathways that contribute to oxidative stress and enzymes involved in the antioxidant defense system (Pérez-Matute *et al.*, 2009).

Mitochondria are the basis of most cellular ROS, precisely superoxide radicals. The reactions that produce ATP in the mitochondria involve electrons from reduced substrates to be delivered along the complexes of the electron transport chain (Pérez-Matute *et al.*, 2009). In the occurrence of molecular oxygen, electrons that seep from this process react and generate superoxide. Superoxide anions are important mediators in many oxidative chain reactions and they are also precursors to many other ROS (Halliwell and Gutteridge, 2015). Other important intracellular causes of ROS production include NADPH oxidases (which generate superoxide), nitric oxide synthases (nitric oxide), and lipoxygenases (fatty acid hydroperoxides). Also, specific cells can stimulate an oxidative environment. For example, macrophages can yield oxidative stress as part of the inflammatory response (Federico *et al.*, 2007). Some ROS, such as superoxide and hydrogen peroxide are essential in supporting natural cellular function and for the regulation of intracellular signaling (Pérez-Matute *et al.*, 2009). However, excess ROS production (or reduced ROS regulation) could cause impairment of cells and subsequently lead to macromolecular damage, dysfunction, and death (Hartley *et al.*, 1997).

Oxidation of DNA has been known to be involved in cellular senescence, apoptosis, and the progression of cancerous cell phenotypes. On the other hand, oxidation of lipids is the basis for changes in structure and fluidity of cellular and organelle membranes that are harmful to cellular processes and functions (Esterbauer *et al.*, 1993). This disturbs the normal cellular functioning, further increasing cellular ROS concentrations. In addition, oxidation of lipids might form lipid radical species that could possibly cause impairment of many cellular macromolecules. For example, lipid peroxides like malondialdehyde (MDA) and 4-hydroxynonenal (4HNE) can react with both DNA and proteins (Hartley *et al.*, 1997).

Aerobic organisms have developed a composite antioxidant system comprising of general antioxidants (that is, those that reduce oxidative stress by removal of ROS) and specialized enzymes that can repair some forms of oxidation within cellular macromolecules (Halliwell and Gutteridge, 2015). These complex processes were developed, since oxidative stress could cause lethal effects even under normal physiological conditions. There are several enzymes involved in the antioxidant defense system and they take part in special roles in defense and repair. Superoxide dismutase (SOD) decreases superoxide levels in the cell; these enzymes catalyze the conversion of the superoxide radical to molecular oxygen and hydrogen peroxide. They are one of the first lines of defense against superoxide radicals produced by the mitochondria during cellular respiration and against superoxide produced by other cellular sources such as NADPH oxidases (Halliwell and Gutteridge, 2015).

Peroxides, including those generated by SOD are converted into water in the cell primarily by catalase, glutathione peroxidases, and peroxiredoxins. Catalase (CAT) is ubiquitously expressed among mammalian tissues and is primarily located in the peroxisomes. The primary catalytic function of catalase is the decomposition of hydrogen peroxide to oxygen and water (Halliwell and Gutteridge, 2015). In general, glutathione peroxidases (GPx) can reduce peroxides (including hydrogen peroxide and lipid hydroperoxides) to less toxic forms including water and alcohols. In addition, thioredoxins (Trx) catalyze reduction of disulfide bonds in multiple substrate proteins (Halliwell and Gutteridge, 2015). Through this reaction, Trxs act as antioxidants by detoxifying peroxides through peroxiredoxins and by reducing protein disulfides and methionine sulfoxides, either directly or through the actions of other oxidoreductases (Powis and Montfront, 2001).

1.2.2. Oxidative stress and insulin resistance

Excessive oxidative stress is detrimental to insulin signaling. There are now several lines of evidence from both model organisms and clinical studies that identified a strong correlation between insulin resistance, T2DM, metabolic syndrome and oxidative stress. Hyperglycemia itself can cause an increase in oxidative stress as the level of lipid peroxidation in erythrocytes was found to be directly proportional to the glucose concentrations *in vitro* and to blood glucose concentrations in diabetic patients (Rain and Jain, 2011). Diabetes is associated with reduced levels of components of the antioxidant defense system including GSH, vitamin E, and vitamin C (Rains and Jain, 2011). Diabetes and insulin resistance are also associated with reduction in total antioxidant activity, SOD, GPx, and glutathione reductase activity. Hyperglycemia may be a significant factor in the down regulation of antioxidant activity.

Oxidative stress could lead to insulin resistance by stimulating the expression of several proinflammatory cytokines (Perez *et al.*, 2009). Several research groups have found an association between increased carbonylation and nitrosylation of proteins in insulin-sensitive tissues and obese or insulin-resistant phenotypes (Perez *et al.*, 2009). Oxidation of specific proteins *in vitro* has a clear detrimental effect on their function. And there is a strong correlation between increasing oxidative stress and diminished protein folding and function in different animal models (Perez *et al.*, 2009). It might be predicted that oxidation of proteins is important for insulin signaling which could potentially affect their function in propagation of insulin-stimulated signals (Perez *et al.*, 2009).

In vitro studies have shown that oxidative stress can impair the ability of insulin receptor to correctly bind with insulin (Fridlyand and Philipson, 2006). Oxidative stress can also alter the ability of insulin signaling proteins to redistribute to correct subcellular locations that are necessary for efficient transduction of signals. From a therapeutic standpoint, it is evident that reduced oxidative stress may be beneficial in the prevention and treatment of insulin resistance. Insulin resistance significantly affects the carbohydrate metabolism as well as blood glucose regulation.

1.3. Carbohydrate metabolism and blood glucose regulation

One of the key dietary sources of glucose in the human body is carbohydrates from starch, which is also a product of starch hydrolysis. Carbohydrates have an influential impact on postprandial blood glucose levels after being absorbed in the small intestine (Dhital *et al.*, 2013). The major link among diabetes, obesity and other metabolic disorders are correlated to the rate and extent of carbohydrate (CHO) digestion and the absorption of glucose (Dhital *et al.*, 2013).

The CHO is hydrolyzed into oligosaccharides, disaccharides and monosaccharides by CHO hydrolyzing enzymes such as alpha amylase (AA) and alpha glucosidase (AG). Alpha amylase consists of a group of calcium dependent enzymes that catalyzes the random break down of amylose and amylopectin units in starch to yield smaller CHO units like maltotriose, maltose and glucose (Qian *et al.*, 1997). In humans, they are found in several tissues, but are mostly present in the saliva and pancreatic juice (Qian *et al.*, 1997).

Alpha glucosidase catalyzes the release of alpha glucose from the reducing terminal end of alpha glycosyl residue-possessing substrates (Okuyama *et al.*, 2005). In human, they are present in the intestinal mucosa as two membrane bounded protein complexes (maltase-glucoamylase and sucrase-isomaltase), which catalyzes from the reducing, end the hydrolysis of α -1,4-glycosidic linkages resulting from AA starch degradation to form free glucose (Dhital *et al.*, 2013).

After the starch and carbohydrates have been broken down into glucose, it is thereafter absorbed into the blood by the wall of the duodenum. The body consists of correct mechanisms to confirm physiological blood glucose homeostasis. When glucose is not required, it is removed from the blood by the conversion of the excess glucose to glycogen, which is then stored in the liver. When the body needs energy or when the blood glucose levels have decreased, the glycogen which is stored in the liver is broken down into glucose and released

into the bloodstream. (Mathews *et al.*, 2000). Glucagon and insulin are the two major hormones in the body that work consistently to regulate and maintain physiological blood glucose homeostasis.

When the blood glucose levels are low, the alpha cells found in the pancreas produces glucagon. Glucagon hormone causes the cAMP dependent activation of glycogen phosphorylase, which then catalyzes the phosphorylytic breakdown of glycogen to release glucose-6-phosphate into the blood (Mathews *et al.*, 2000). When the glycogen levels are low, the glucagon hormone stimulates gluconeogenesis. Insulin is released from the beta-cells of the pancreas, when the glucose levels in the blood rises due to the breakdown of glycogen or intestinal glucose absorption, and this promotes cell glucose uptake and by the cell membrane glucose transporter type 4 (GLUT4); glycolysis; inhibition of glucagon secretion by alpha cells; and also signals the activation of glycogen synthase that catalyzes the conversion of glucose to glycogen, thus normalizing blood glucose level (Mathews *et al.*, 2000).

Insulin resistance is a disorder in glucose metabolism and homeostasis that can give rise to hyperglycemic conditions in T2D.

1.4. Insulin and normal insulin signaling

Insulin is an important hormone that plays a role in part in the growth and development of tissues and the control of glucose equilibrium in the body (Rains and Jain., 2011). It is released by pancreatic β -cells and the prime role of this hormone is to control glucose homeostasis by the stimulation of glucose transport into muscle and adipose cells, while reducing hepatic glucose production via gluconeogenesis and glycogenolysis (Rains and Jain, 2011). Another important role of insulin, is that it can regulate lipid metabolism by increasing lipid synthesis in liver and fat cells while inhibiting lipolysis. Insulin is also essential for the uptake of amino acids and protein synthesis (Rains and Jain, 2011). However, insulin resistance one of the two major pathogenesis of type 2 diabetes.

1.4.1. Insulin resistance

Insulin resistance is associated with the onset and development of T2D. It is linked with various factors such as genetic and environmental factors. Nonetheless, environmental factors like lack of exercise, aging, high carbohydrate and high fat diet and obesity are the most impelling factors which can contribute to the progression and development of insulin resistance (Kahn, 2003). Insulin resistance transpires when affected cells such as myocytes, adipocytes and hepatocytes do not efficiently react to insulin hormone signaling components. Cells become

insensitive to insulin and therefore the release of insulin hormone into the blood does not efficiently regularize the level of blood glucose (Kahn, 2003).

Insulin is also significant in the regulation of body fat metabolism. It is critical for promoting uptake of circulating free fatty acid (FFA); differentiation and maturation of adipocytes; lipogenesis; and inhibition of lipolysis (Kahn, 2003). Consequently, a deficiency in fat metabolism, abnormally high plasma FFA and triacylglyceride and irregularly low plasma high density lipoprotein levels can transpire during insulin resistance, which can influence an insulin resistant patient to obesity, metabolic syndrome and other complications related to diabetes (Rabøl *et al.*, 2011). Many cellular studies reported and shown that under oxidative stress conditions, insulin signaling is impaired, which results in insulin resistance. This could be investigated by measuring glucose uptake (Rains and Jain, 2011).

1.5. Intestinal absorption of glucose

Persistent hyperglycemia throughout type 2 diabetes is intensely influenced by the degree of intestinal absorption of glucose resulting from digestible carbohydrate like starch and sucrose consequently, by limiting the rate of postprandial intestinal glucose absorption will transform into significant glycemic control (Hanhineva *et al.*, 2010).

Intestinal absorption of glucose is mediated by active transport via sodium-dependent glucose transporter (SGLT1) and by facilitated sodium-independent transport via the glucose transporter GLUT2 (Hanhineva *et al.*, 2010). The reduction of late postprandial glycemia may be explained by an elevated insulin response following stimulation of pancreatic beta-cells rather than by retarded absorption of glucose (Hanhineva *et al.*, 2010).

In the recent years, type 2 diabetic patients are always trying to reduce their blood glucose levels not only by taking oral hypoglycemic drugs but also by using various functional and medicinal foods and food supplements, caffeine is one of them in our regular food list.

1.6. Caffeine

Caffeine (1, 3, 7-trimethylxanthine) is an alkaloid belonging to the methylxanthine family. It is naturally present in the seeds, leaves and fruits of more than 63 plant species (Del Coso *et al.*, 2012; Geethavani *et al.*, 2014). Approximately 80% of the world population consumes at least one caffeinated product daily (Kumar *et al.*, 2018). Caffeine consumption is most common through beverages such as coffee (71%), soft drinks (16%) and tea (12%) (Nehlig, 1999). Different countries consume caffeine at various levels around the world, with an average

of more than 300 mg/day in leading countries like Denmark, Finland and Brazil (Kumar *et al.*, 2018).

1.6.1. Natural sources of caffeine

The most important sources of caffeine are coffee (*Coffea* spp.), tea (*Camellia sinensis*), guarana (*Paullinia cupana*), kola nuts (*Cola vera*), cocoa (*Theobroma cacao*) and caffeinated soft drinks which includes energy drinks (Fulgoni *et al.*, 2015). Coffee is cultivated globally, and the key producers are Brazil, Vietnam, Indonesia, Columbia and Ethiopia (Pohlan and Janssens, 2010). Whereas, tea is the most extensively ingested beverage after water and contains caffeine as an active dietary constituent. Guarana, a product of a climbing shrub (*Paullinia cupana*) is native to Brazil, Bolivia, Peru, Uruguay and Venezuela and being rich in caffeine, guarana and kola nuts relish great attractiveness as they serve as a stimulant (Muhammad and Fatima, 2014). Several caffeinated products are available in the market which is attracting consumer's attention. Caffeine is utilized for the preparation of many coffee and coffee-based beverages, tea, energy drinks and cocoa products.

Caffeine is absorbed in the body thereafter it reaches the brain within five minutes after ingestion (Baribeau *et al.*, 2014) and is also eliminated with an average half-life of five hours from the body (Heckman *et al.*, 2010). Once ingested, caffeine is rapidly absorbed from the gastrointestinal tract into the bloodstream and becomes metabolized in the liver (Heckman *et al.*, 2010). Caffeine is metabolized by the liver to form 3 major metabolites, which are 3,7-dimethylxanthine, 1,7-dimethylxanthine, and 1,3-dimethylxanthine. Once absorbed, caffeine employs many physiological actions to various organs in the body.

1.6.2. Beneficial effects of caffeine

Caffeine has many pharmacological as well as physiological effects on the human body. Some of these effects are on respiratory (Franco *et al.*, 2013), cardiovascular (Butt and Sultan, 2013), gastrointestinal and the central nervous system (Baribeau, 2014). Caffeine is commonly consumed for its role to work as a mild nervous chemical stimulant towards drowsiness and fatigue (Wolde, 2014). It is used as an adjuvant analgesic in combination with drugs like acetaminophen, aspirin and ibuprofen (Migliardi *et al.*, 1994).

The various beneficial effects of it include the reduced risk of diabetes by exerting positive effects on glucose metabolism and liver injury (Wolde, 2014), It helps to reduce the risk of Parkinson's disease and lowers the risk of Alzheimer's disease (Messina *et al.*, 2015), improve

psychomotor performance and improves overall immune response (Wolde, 2014). Caffeine offers diverse benefits including its role in increasing alertness, vigilance, mood as well as psychomotor and cognitive performance (Glade, 2010).

1.6.3. Caffeine and its role as an antioxidant

Various biochemical reactions in our body create ROS essentially comprising free radicals that have detrimental effects on biomolecules. Oxidative stress results when they are not effectively neutralized by the antioxidant defense systems. Polyunsaturated fatty acids have become a central area of interest in the biochemistry of oxidative stress caused by ROS (Sies, 1986). These fatty acids are mainly present in the phospholipids of biological membranes and their unspecific oxidation by ROS known as lipid peroxidation is a radical mediated pathway and a highly damaging event. It generates toxic byproducts such as 4-hydroxy- nonanal known to alter other biomolecules in the cell (Buettner, 1993). One of the areas which have attracted a great deal of attention is the possible role of antioxidants in the control of oxidative damage. Therefore, it helps to prevent different diseases by protecting cells in the body against oxidative damage (Wolde, 2014) and raise the overall immunity of a person. Studies have shown that caffeine, at millimolar concentrations, is a potent antioxidant capable of preventing lipid peroxidation. These reports have suggested that the ability of caffeine to inhibit lipid peroxidation is similar to glutathione and significantly greater than that of ascorbic acid.

1.6.4. Caffeine and its role in diabetes

Several reports have demonstrated that the consumption of Coffee (a source of caffeine) is linked with a lower risk of type 2 diabetes mellitus (Heckman *et al.*, 2010). Caffeine intake has been found to decrease one's sensitivity towards insulin, leading to decreased storage of glucose (Butt and Sultan, 2011). Caffeine exerts positive effects on glucose metabolism through increased uncoupling protein expression and lipid oxidation leading to decreased glucose storage capacity which in turn reduces the degree of diabetes mellitus (Butt and Sultan, 2011). A report shows that high caffeine intake is related with reduction in diabetes risk. (Wolde, 2014).

It is also reported that caffeine improved peripheral glucose utilization with insulin sensitivity, improved insulin secretion and increased glucokinase expression as well as increased pancreatic β -cell mass by increased proliferation in diabetic rats (Park *et al.*, 2007). Caffeine has beneficial effects on peripheral insulin sensitivity (Park *et al.*, 2002) and has

neuroprotective effects in STZ-induced diabetic rats by preventing synaptic degradation and astrogliosis in the brain which is caused by hyperglycemia (Duarte *et al.*, 2009).

It is postulated that caffeine has ability to inhibit adenosine monophosphate phosphodiesterase (cAMP-PDE) which leads to an increase in intracellular cAMP concentrations (Horrigan *et al.*, 2008). Intracellular accumulation of cAMP enhances glucose regulation in the pancreatic β -cells and liver (Horrigan *et al.*, 2008). by activating the cAMP-responding element-binding protein which results in an increase in IRS-2 expression. This leads to an improved glucose homeostasis by enhancing insulin and insulin-like growth factor 1(IGF-1) signaling in the islets. This insulin signaling process occurs through the activation of tyrosine phosphorylation which activates Akt that is located downstream of IRS-2 (Jhala *et al.*, 2003).

In 2008, Kagami *et al.* observed that caffeine has a potent pancreatic β -cell protecting effect in rat and they found that the pancreatic insulin content was significantly recovered by caffeine pre-treatment at 100 mg/kg body weight (Kagami *et al.*, 2008).

1.6.5. Caffeine and weight management

Caffeine supplementation has been recently considered as an effective means of weight management. It increases metabolism and thus plays an effective role in weight management (Heckman *et al.*, 2010). Oxygen consumption and fat oxidation was found to increase after caffeine consumption which suggests its role in weight loss (Greenway, 2001). Another report showed that caffeine consumption, as contained in green tea, has been associated with weight reduction (Wedick, 2011). Energy balance is the main determinant of weight regulation. Research on caffeine has confirmed its role in increasing metabolic rate, energy expenditure (EE), lipid oxidation, and lipolytic and thermogenic activities; all favorable components regarding weight management and possible weight loss in humans (Greenway, 2001).

1.7. Rationale of the study

In addition to lifestyle modifications, there are several therapeutic approaches used in the management of T2D and related disorders like oxidative stress. These include synthetic anti-diabetic drugs like sulphonylureas (glibenclamide), glucosidase inhibitors (acarbose) and biguanide (metformin) as well as natural and synthetic anti-oxidants. However, most synthetic oral hypoglycemic agents have reportedly shown characteristic profiles of serious side effects such as hypoglycemia, weight gain, gastrointestinal discomfort and nausea, liver and heart failures and diarrhea. Hence, there has been a need to explore other therapeutic agents to replace or synergistically complement the existing ones.

1.8. Aim

To investigate the anti-diabetic mechanisms of caffeine in a type 2 diabetes model of rats.

1.9. Objectives of the study

- To investigate the *in vitro* and *ex vivo* anti-oxidative activities of caffeine.
- To investigate the inhibitory effect of caffeine on α -amylase, α -glucosidase and pancreatic lipase activities.
- To investigate the effect of caffeine on intestinal glucose absorption in isolated rat jejunum and muscle glucose uptake in rat psoas muscle.
- To investigate the *in vivo* anti-oxidative and anti-diabetic mechanisms of caffeine in a type 2 diabetes model of rats.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals and reagents

Ascorbic acid, calcium chloride di-hydrate, caffeine, dinitrosalicylic acid (DNS), disodium EDTA, ferric chloride, ferrous sulphate heptahydrate, Griess reagent, hydrogen peroxide, mono-basic mono-basic potassium phosphate, sodium chloride, sodium bicarbonate, sodium citrate, sodium hydroxide, sodium hydrogen carbonate, sodium phosphate, sodium nitroprusside, starch, sulphuric acid, trichloroacetic acid, reduced glutathione and thiobarbituric acid were purchased from Merck Chemical Company, Durban, South Africa.

Acarbose, ammonium molybdate tetrahydrate, citric acid, 2 deoxy-D-ribose, di-basic sodium phosphate, diethylenetriaminepentaacetic acid (DETAPAC), 5,5'-dithiobisnitrobenzoic acid (DTNB), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 6-hydroxydopamine, p-nitrophenyl- α -D-glucopyranoside (pNPG), 3-(N-morpholino) propanesulfonic acid (MOPS) p-nitrophenol, porcine pancreatic amylase, potassium ferricyanide, sodium acetate, streptozotocin and yeast α -glucosidase, were obtained from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Glucose and fructose were bought from Associated Chemical Enterprise (ACE), South Africa, while metformin was purchased from a local pharmacy (Pharmed) in Durban, South Africa.

An ultra-sensitive rat insulin ELISA kit was purchased from Mercodia AB, Uppsala, Sweden. Reagents to test the other serum parameters were bought from Labtest Diagnostics (Lagoa Santa, Brazil) purchased through Replamed Company Ltd., Centurion, South Africa.

2.2. Equipment

Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), Steri-Cult CO₂ incubator (Labotec, South Africa), Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Lagoa Santa, Brazil), Glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada), Ultra Turrax Tube Drive Work Station Homogenizer (IKA-Works, Staufenim Breisgau, Germany), Hettich Mikro 200 Microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany), Synergy HTX Multi-mode Reader (BioTek Instruments Inc, Winooski, USA), Microfuge 20R Centrifuge (Beckman Coulter, Inc., Germany), Eppendorf™ 5810R Centrifuge (Fisher Scientific, UK).

2.3. *In vitro* Studies

2.3.1. DPPH radical scavenging activity

Principle: DPPH (1, 1- Diphenyl-2-picrylhydrazyl) is a stable free radical with red color (maximum absorbance at 517 nm). If free radicals have been scavenged, DPPH will change the red color to yellow.

Procedure: The free radical scavenging activity of caffeine was determined and comparison was made to gallic acid, ascorbic acid and trolox according to the method described by (Ak and Gülçin 2008). Briefly, 80 µL of a 0.3 mM solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) in distilled water was mixed with 170 µL of caffeine at different concentrations (15, 30, 60, 120, 240 µg/mL). These solutions were incubated at dark for 30 min at room temperature. The absorbance of the solutions was measured at 517 nm against blank, lacking the free radical scavenger.

All assays were carried out in triplicate. The DPPH radical scavenging activity was measured by using following formula:

$$\% \text{ inhibition} = \left[\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \right] \times 100$$

2.3.2. Ferric reducing antioxidant power assay (FRAP)

Principle: The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced oxidant, Fe (III). Reduction of a ferric (III) [colorless] to ferrous (II) [blue] can be measured photometrically. The absorbances are related to the reducing power of the electron-donating antioxidants present in the test sample.

Procedure: Briefly, 300 µL of caffeine at different concentrations (15, 30, 60, 120, 240 µg/mL), were incubated with 300 µL of sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide in a water bath at 50°C for 30 min. Thereafter, the solution was acidified with 300 µL of 10% trichloroacetic acid and 300 µL of this sample was then added to 300 µL of distilled water and 0.1 µL of 0.1% FeCl₃. The absorbance of the solution was measured at 700 nm and the results were expressed as gallic acid equivalent.

All assays were carried out in triplicate. The percent inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = \left[\frac{\text{absorbance of sample}}{\text{absorbance of gallic acid}} \right] \times 100$$

2.3.3. Nitric oxide (NO*) radical scavenging assay

Principle: Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions that can be measured by using Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of NO (Kurian *et al.*, 2010).

Procedure: The assay was carried out by incubating 500 µL of 10 mM sodium nitroprusside in phosphate buffer (pH 7.4) solution and 500 µL of caffeine with different concentrations (15, 30, 60, 120, 240 µg/mL), at 37°C for 2 h. The reaction mixture was then mixed with 500 µL of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm. Percentage inhibition of nitric oxide generated was measured by comparing the absorbance of control (10 mM sodium nitroprusside in phosphate buffer) with the absorbance of sample.

All assays were carried out in triplicate. The nitric oxide radical scavenging power of the samples was calculated by using following formula:

$$\% \text{ inhibition} = \left[\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \right] \times 100$$

2.3.4. Hydroxyl radical (OH*) scavenging assay (Deoxyribose method)

Principle: Hydroxyl radical scavenging activity of caffeine was measured by studying the competition between deoxyribose and caffeine for hydroxyl radical generated by ascorbate–EDTA–H₂O₂ system (Fenton reaction) as described by (Hinneburg, Dorman *et al.* 2006).

Procedure: The assay was performed by sequentially adding 200 µL of premixed 100 µM FeCl₃ and 100 µM EDTA (1:1 v/v) solution, 100 µL of 10 mM H₂O₂, 360 µL of 10 mM 2-deoxy-D-ribose, 1 mL caffeine with different concentrations (15, 30, 60, 120, 240 µg/mL), 400 µL of 50 mM sodium phosphate buffer (pH 7.4) and 100 µL of 1 mM ascorbic acid in sequence. The mixture was then incubated at 50°C for 2 h. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% thiobarbituric acid (in 0.025 M NaOH) were added to each tube. The samples were further

incubated in a water bath at 50°C for 30 min to develop the pink chromogen. The extent of oxidation was estimated from the absorbance of the solution at 532 nm and the hydroxyl radical scavenging activity of the sample is reported as percentage inhibition of deoxyribose degradation.

All assays were carried out in triplicate. The scavenging activities of caffeine hydroxyl radical scavenging assay was calculated by using the following formula:

$$\text{Scavenging Activity (\%)} = \left[\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \right] \times 100$$

2.3.5. Measurement of *in vitro* alpha amylase inhibitory activity

Principle: Alpha amylase activity can be measured by the hydrolysis of starch in the presence of α -amylase enzyme. This process is quantified using iodine, which gives a blue colour with starch. The reduced intensity of the blue colour indicates the enzyme-induced hydrolysis of starch into monosaccharides. If the sample compound possesses more α -amylase inhibitory activity, the intensity of blue colour will be less.

Procedure: The α -amylase inhibitory activity was determined according to the method as described by (Shai, Masoko *et al.* 2010). Briefly, 250 μ L of caffeine or acarbose at various concentrations (15, 30, 60, 120, 240 μ g/mL) was incubated with 500 μ L of porcine pancreatic amylase (2 U/mL) in 100 mM phosphate buffer (pH 6.8) at 37°C for 20 min. Thereafter, 250 μ L of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was added to the reaction mixture and incubated at 37°C for 1 hour. Dinitrosalicylate colour reagent (1 mL) was then added and boiled for 10 min. The absorbance of the resultant mixture was measured at 540 nm and the inhibitory activity was expressed as percentage of a control without the inhibitors (sample compound or standard, acarbose).

All assays were carried out in triplicate. The enzyme inhibitory activity of the sample compound or standard acarbose has been calculated according to the following formula:

$$\% \text{ inhibition} = \left[\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \right] \times 100$$

2.3.6. Measurement of *in vitro* alpha glucosidase inhibitory activity

The alpha glucosidase inhibitory activity was determined according to the method described by (Ademiluyi and Oboh 2013) with slight modifications. Briefly, 250 µL of caffeine or acarbose at different concentrations (15, 30, 60, 120, 240 µg/mL) were incubated with 500 µL of 1.0 U/mL α-glucosidase solution in 100 mM phosphate buffer (pH 6.8) at 37°C for 15 min. Thereafter, 250 µL of pNPG solution (5 mM) in 100 mM phosphate buffer (pH 6.8) was added and the mixture was further incubated at 37°C for 20 min. The absorbance of the released p-nitrophenol was measured at 405 nm and the inhibitory activity was expressed as percentage of the control without the inhibitors.

All assays were carried out in triplicate. The α-glucosidase inhibitory activity was calculated in percent (%) as per following formula:

$$\% \text{ inhibition} = \left[\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \right] \times 100$$

2.3.7. *In vitro* pancreatic lipase inhibitory assay

The method for measuring pancreatic lipase inhibitory activity of sample compound or standard (Orlistat) was measured by using a slightly modified method from (Kim, Lee *et al.* 2010). Briefly, an enzyme buffer was prepared by the addition of a solution of porcine pancreatic lipase [2.5mg mL⁻¹ in 10 mM MOPS (morpholinepropanesulphonic acid) and 1 mM EDTA, pH 6.8]. Thereafter 169 µL of Tris buffer (100 mM Tris-HCl and 5 mM CaCl₂, pH 7.0) was added to 100 µL of caffeine (15, 30, 60, 120, 240 µg/mL) at the test concentration, or orlistat, after which 20 µL of the enzyme buffer was added and incubated 15 min at 37°C. A 5 µL of the substrate solution [10 mM p-NPB (p-nitrophenyl butyrate) in dimethyl formamide] was then added and incubated for 30 min at 37°C. The lipase activity was determined by measuring the hydrolysis of p-NPB to p-nitrophenol at 405 nm.

The lipase inhibitory activity of caffeine on lipase were calculated by using the following formula:

$$\% \text{ inhibition} = \left[\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \right] \times 100$$

2.4. *Ex vivo* studies

Animal procedures

Five adult male Sprague-Dawley rats were procured from the Biomedical Resource Centre located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. The animals were fasted over-night (12 h) and euthanized with halothane anesthesia. The pancreas, the entire gastrointestinal tract (GIT) and parts of the psoas muscle were harvested after dissection and instantly used for antioxidative stress assays, glucose absorption and muscle glucose uptake studies respectively. All animal procedures were carried out according to the rules and regulations of the Animal Ethics Research Committee of University of KwaZulu-Natal, South Africa (Ethical approval number: AREC/003/017D).

2.4.1 *Ex vivo* antioxidative studies

➤ Preparation of tissue homogenate

Half a gram (0.5 g) of freshly excised pancreatic tissue was homogenized in 5 mL of homogenization buffer (50mM sodium phosphate buffer with 10% Triton X-100, pH 7.5). The homogenate was centrifuged for 15 min at 15,000 rpm in a micro centrifuge. The supernatant was collected and stored at -20°C for further analysis.

➤ Induction of tissue oxidative stress and treatment

Oxidative stress was induced in pancreatic tissue as described by Oboh *et al.*, 2013. A 30 μL of 15mM ferrous phosphate (FeSO_4) was incubated with 100 μL of different concentration (15, 30, 60, 120, 240 $\mu\text{g/mL}$) of the compound and standard (gallic acid) and the pancreatic tissue homogenate for 30 min at 37°C . A reaction containing no compound and ferrous phosphate was served positive control (normal) while reaction with no compound was served as a negative control (untreated).

2.4.1.1 Determination of lipid peroxidation

Lipid peroxidation was carried out according to the method of Oboh *et al.*, 2013, with slight modifications. A 200 μL of 8.1% of SDS solution, 750 μL of 20% acetic acid, 2 mL of 0.25% TBA solution and 850 μL of miliQ water were added to the solution containing tissue homogenate and boiled for an hour. After cooling, the absorbance was measured at 532 nm. MDA concentrations of samples were calculated from the MDA standard curve.

2.4.1.2 Determination of reduced glutathione (GSH) activity

Determination of GSH activity was carried out according to the method described by Ellman *et al.*, 1959. 100 μ L of the incubated sample was added to 200 μ L of 10% TCA and centrifuged at 3500 rpm for 5 min. Thereafter, 200 μ L of the supernatant was added to a 96-well plate and the absorbance was measured at 415 nm. GSH concentrations of samples were calculated from the GSH standard curve.

2.4.1.3 Determination of superoxide dismutase (SOD) enzyme activity

Briefly, 170 μ L of 0.1mM diethylenetriaminepentaacetic acid (DETAPAC) and 15 μ L of the solution were placed in a 96-well plate. Then 15 μ L of 1.6mM 6-hydroxydopamine (6-HD) was added and the mixture was quickly mixed by gently tapping all four sides of the plates. Absorbance was recorded at 492 nm for 3 min at 1 min interval. SOD enzyme activity was calculated using the following formula:

$$Activity = \frac{(A1 - Ab)}{\epsilon_{490} \times RV \times Df / Sv}$$

ϵ_{490} = Molar absorptivity at 490 nm = 1.742/mM/cm

A1 and Ab = Reaction rate for sample and blank respectively

RV = Reaction Volume; Df = Dilution factor; Sv = Sample volume

2.4.1.4 Determination of catalase activity

Catalase activity was determined using a spectrophotometric molybdate method as described by (Chance *et al.*, 1955). Briefly, 100 μ L of the incubated tissue homogenate was mixed and incubated at 37 °C with 1000 μ L of 65 μ M H₂O₂ in 6.0mM sodium phosphate buffer, pH 7.4 for 2 min. The reaction was stopped by adding 4000 μ L of 32.4mM of ammonium molybdate and the color change in the yellowish molybdate/H₂O₂ complex was measured at 347 nm against the reagent blank that contains only H₂O₂. The standard tube contained all the reagents except the tissue sample, and the control test tube contained all other reagents except H₂O₂. Catalase activity was calculated using the following formula:

$$CAT \text{ activity (kU)} = 2.303/t * \log [So/(S-M)] * Vt/Vs$$

t: time, S°: absorbance of standard tube, S: absorbance of test tube, M: absorbance of control test, Vt: total volume of reagents in test tube, Vs: volume of test sample.

2.4.2 Measurement of glucose absorption in isolated rat jejunum

The effect of caffeine on glucose absorption in isolated rat jejunum (proximal part of small intestine) was determined by using method as modified from Hassan *et al.*, (2010) and described in the following sections.

➤ Preparation of reagents

Preparation of 1 L Kreb's buffer: Kreb's buffer is composed of 118 mM sodium chloride (NaCl), 5 mM potassium chloride (KCl), 1.328 mM calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 1.2 mM potassium mono-basic phosphate (KH_2PO_4), 1.2 mM magnesium sulphate (MgSO_4) and 25 mM sodium hydrogen bi-carbonate (NaHCO_3). A 1 L of Kreb's buffer was prepared by dissolving 6.896 g of NaCl (M.W., 58.44 g/mol), 0.373 g of KCl (M.W., 74.56 g/mol), 0.188 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (M.W., 147.02 g/mol), 0.163 g of KH_2PO_4 (M.W., 136.09 g/mol), 0.144 g of MgSO_4 (M.W., 120.37 g/mol) and 2.10 g of NaHCO_3 (M.W., 84.01 g/mol) in 1 L of autoclaved dH_2O .

Preparation of 500 mL of 11.1 mM glucose Kreb's solution (GKS): This was prepared by dissolving 0.9999 g of D-glucose (M.W., 180.16 g/mol) in 500 mL of Kreb's buffer.

2.4.2.1 Assay procedure

The assay was performed in three replicates. Five 5 cm length of jejunum was cut from isolated GIT of rats and each jejunum was rinsed by injecting 2 mL of Kreb's buffer through the jejunal lumen using a sterile syringe. Rinsed jejuna were first inverted to expose their inner wall and villi, and then incubated in incubation tubes containing 8 mL each of GKS solution without (control) or with caffeine at different concentrations (60, 120, 240 $\mu\text{g/mL}$) for 2 h in a Steri-Cult CO_2 incubator (Lasec, South Africa) at 5% CO_2 , 95% oxygen and 37°C condition. A 1 mL aliquot was collected from each incubation solution before and after incubation and the glucose concentrations were measured in milligram per deciliter (mg/dL) with Thermo Scientific glucose kit in a Labmax Plenno Chemistry Analyzer (Labtest Inc., Lagoa Santa, Brazil).

2.4.2.2 Calculation of intestinal glucose absorption

The absorption of intestinal glucose was calculated as the amount of glucose (mg) absorbed per centimeter of rat jejunum using the following formula:

$$\text{Intestinal glucose absorption} = [(GC1 - GC2) / \text{length of jejunum taken in cm}]$$

GC1: glucose concentrations (mg/dL) before incubation

GC2: glucose concentrations (mg/dL) after incubation

2.4.3 Measurement of glucose uptake in isolated rat psoas muscles

The effect of caffeine on glucose uptake in isolated rat psoas muscles was determined according to method modified from Abdel-Sattar *et al.*, (2012).

2.4.3.1 Assay procedure

The assay was carried out in triplicates. Three pieces of 0.5 g of psoas muscle was cut from each rat and incubated in incubation tubes containing 8 mL of GKS without (control) or with the different concentrations of caffeine solutions (60, 120, 240 µg/mL) or 1 mg/mL metformin solution (positive control) for 1 h in a Steri-Cult CO₂ incubator (Labotec, South Africa) at 5% CO₂, 95% Oxygen at 37°C condition. A 1 mL aliquot was collected from each incubation tube before and after incubation and the glucose concentrations were measured in milligram per deciliter (mg/dL) by using Thermo Scientific glucose kit in a Labmax Plenno chemistry analyzer (Labtest Inc., Costa Brava, Brazil).

2.4.3.2 Calculation of muscle glucose uptake

The muscle glucose uptake was calculated as the amount of glucose absorbed per gram of rat psoas muscle using the following formula:

$$\text{Muscle glucose uptake} = [(GC1 - GC2) / \text{amount of muscle in gram}]$$

GC1: glucose concentrations (mg/dL) before incubation

GC2: glucose concentrations (mg/dL) after incubation

2.5 In vivo Studies

2.5.1. Experimental animals

Forty male Sprague-Dawley (six-week-old) rats with a mean body weight (BW) of 160-200g were obtained from the Biomedical Resource Unit (BRU) located at the University of KwaZulu-Natal (Westville Campus), South Africa. The rats were maintained as 7 in one large sized polycarbonated cage in a humidity (40-60%) and temperature ($23 \pm 1^{\circ}\text{C}$) controlled room with a 12-hour light–dark cycle. A standard rat pellet diet was supplied *ad libitum* for the entire duration of the experiment. All animal procedures were carried out according to the rules and regulations of the Animal Ethics Research Committee of University of KwaZulu-Natal, South Africa (Ethical approval number: AREC/003/017D).

2.5.2. Animal grouping

The rats were randomly divided into seven groups of seven animals each namely:

- Normal control (NC)
- Diabetic control (DBC)
- Diabetic caffeine low dose - 10 mg/kg BW (DCAFL)
- Diabetic caffeine medium dose - 20 mg/kg BW (DCAFM)
- Diabetic caffeine high dose - 40 mg/kg BW (DCAFH)
- Diabetic metformin – 200 mg/kg BW (DM)

2.5.3. Induction of diabetes

The animals were allowed to acclimatize for one week. During the first 2 weeks of the experiment, the animals in the diabetic groups were supplied with 10% fructose solution *ad libitum* instead of drinking water to induce insulin resistance while the animals in the NC group were supplied with normal drinking water. Thereafter, animals in the diabetic groups were intraperitoneally injected with a low dose of streptozotocin (40 mg/kg body weight) dissolved in 0.1 M citrate buffer (pH 4.5) to induce partial pancreatic β -cell dysfunction, while the animals in NC group were injected with a similar volume of citrate buffer only. One week after the streptozotocin injection, the non-fasting blood glucose (NFBG) levels of all animals were measured in the blood collected from the tail vein by using a portable glucometer, and the animals with NFBG level > 200 mg/dL were considered diabetic. Animals with a NFBG level < 200 mg/dL were excluded from the study.

2.5.4. Intervention trial

After the confirmation of diabetes, the respective dose of caffeine was orally administered five days in a week by using a gastric gavage needle to the rats in DCAFL, DCAFM and DCAFH groups while the rats in controls (NC and DBC) and DM groups were treated with a similar volume of the vehicle and metformin respectively for a 5-week experimental period. During this period, daily food and fluid intakes as well as weekly body weight changes and non-fasting blood glucose (NFBG) concentrations were measured in all animal groups.

2.5.5. Oral glucose tolerance test (OGTT)

The OGTT was conducted in the last week of the 5-week intervention period to measure the glucose tolerance ability of each animal. To perform this test, a single dose of glucose solution (2 g/kg BW) was orally ingested into each animal and the levels of blood glucose were measured at 0 (just before the ingestion of glucose), 30, 60, 90 and 120 min after the dose of glucose.

2.5.6. Collection of blood and organs

At the end of the 5-week experimental period, the rats were euthanized by ISOFOR anesthesia and the blood and organ samples were collected. Cardiac puncture method was used to collect the whole blood from each rat which was immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 min and serum from each blood sample was separated and kept at -30°C for subsequent analysis. The brain, liver, kidney and heart and pancreas were collected from each rat, rinsed with normal saline, wiped with filter paper, weighed and kept at -30°C until further analysis. A small portion of the pancreas sample from each rat was cut and immersed into 10% neutral buffered formalin and stored at room temperature for histopathological analysis.

2.5.7 Principles for TC, TG, HDL and LDL cholesterol determination

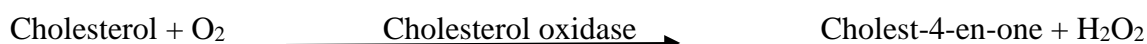
An automated Chemistry Analyzer and biochemical test kits were used to determine the concentration of total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL) cholesterol, low density lipoproteins (LDL) cholesterol, transaminase (AST), alkaline phosphatase (ALP), fructosamine, creatinine and urea in the serum samples.

Total cholesterol (TC):

For total cholesterol (TC) determination, cholesterol esterase hydrolyzes cholesterol esters to yield fatty acids and cholesterol.



Cholesterol oxidase then oxidizes the free cholesterol to cholest-4-en-one and hydrogen peroxide.

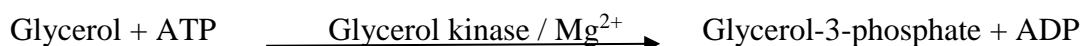
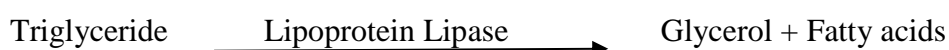


Lastly, phenol and 4-aminoantipyrine are oxidized by peroxidase to yield quinoneimine which has a maximum absorption at wavelength 500 nm. The intensity of the pink colour, due to the end point reaction, is directly proportional to the concentration of cholesterol in the serum samples.



Triglyceride (TG):

For triglyceride (TG) determination, triglycerides are hydrolyzed by lipoprotein lipase to yield glycerol which in turn is converted to glycerol-3-phosphate by glycerolkinase and further oxidized to dihydroxyacetone and hydrogen peroxide by the enzyme glycerolphosphate oxidase.



HDL-cholesterol:

The HDL cholesterol test involves the selective precipitation of LDL cholesterol and very low-density lipoproteins (VLDL) cholesterol from the serum samples using phosphotungstic acid and magnesium chloride. This is obtained by centrifugation and the concentration of HDL cholesterol in the serum samples is measured in the resulting supernatant using the total cholesterol test as explained above.

LDL-cholesterol:

The concentration of LDL cholesterol in the serum samples were determined using the following formula below:

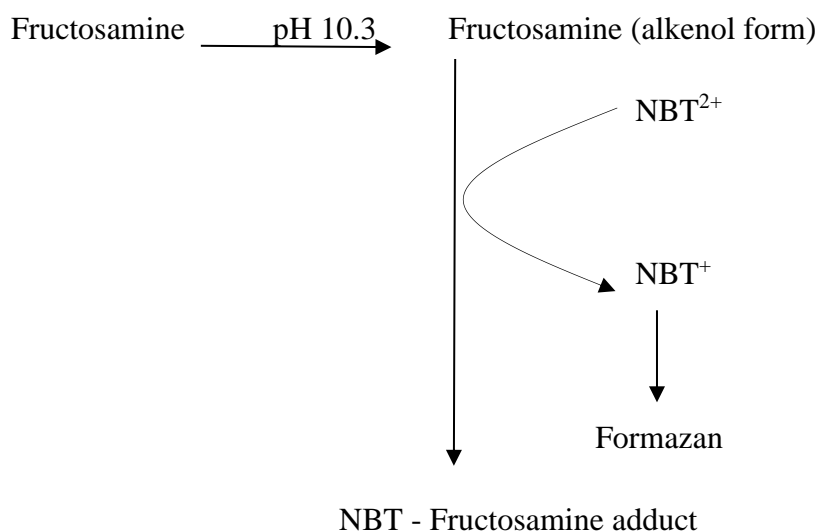
$$\text{LDL cholesterol} = \{ \text{Total cholesterol} - (\text{HDL cholesterol} + \text{VLDL cholesterol}^*) \}$$

$$^*\text{VLDL cholesterol} = \text{Triglycerides} / 5$$

2.5.8 Principles for fructosamine, creatinine and urea determination

Fructosamine:

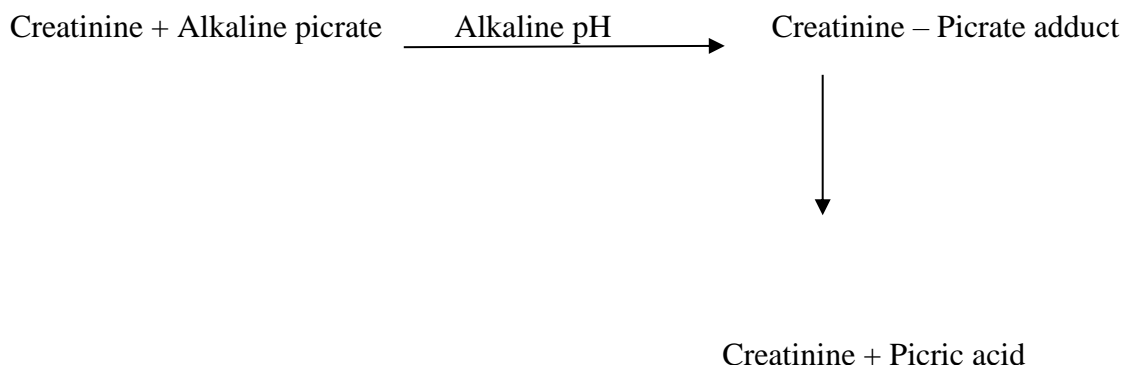
When glucose binds to the amino groups of proteins it yields a Schiff's base (aldimine). It undergoes molecular rearrangements and transforms into fructosamine which is a stable ketoamine (Kyle *et al.*, 2008). In this fixed-time kinetic method test, fructosamine is converted to an enolic form at an alkaline pH of 10.3 and reduces nitroblue tetrazolium (NBT) to a "purple formazan". The difference in absorbance values at 530 nm after incubation of samples with nitro blue tetrazolium for 10 and 15 minutes is proportional to the fructosamine concentration in the serum samples.



Creatinine:

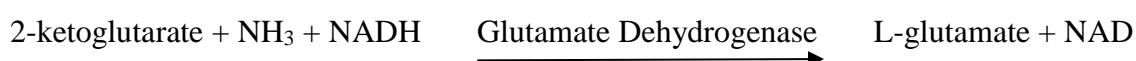
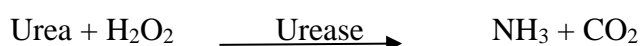
The concentration of creatinine in serum samples was determined by a colorimetric method. Creatinine reacts with alkaline picrate in an alkaline solution thus yielding a red complex that is measured photometrically at 510 nm. When acetic acid is added the pH decreases to 5.0 and the decomposition of creatinine picrate occurs. As a result, the chromogen derived colour

remains unchanged and it is also read photometrically. The differences in absorbance measurements yields a true value of creatinine in the sample.



Urea:

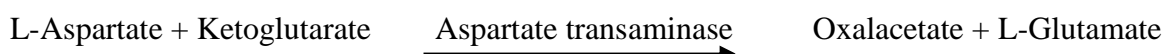
Urea is determined by an enzymatic two-point, fixed-time kinetic method. This test involves the breakdown of urea to ammonia by the enzyme urease. Ammonia is then reacted with NADH and 2-ketoglutarate, catalyzed by glutamate dehydrogenase, which leads to the oxidation of NADH to NAD and the formation of L-glutamate. The resulting reduction in absorbance is measured at 340 nm and it is proportional to the concentration of urea in the samples.



2.5.9 Principles for ALP, AST determination

Aspartate transaminase (AST):

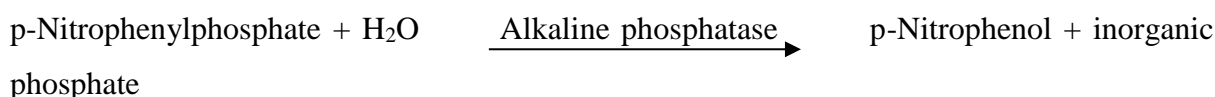
Aspartate transaminase (ASAT) plays a role in catalyzing the transfer of amino groups from aspartic acid to ketoglutarate which yields oxalacetate and glutamate. Malate dehydrogenase reduces oxalacetate to malate and oxidizes NADH to NAD. Oxidation of NADH causes a reduction in absorbance at 340 nm and it is monitored photometrically since it is directly proportional to the activity of ASAT in the serum samples.





Alkaline phosphatase (ALP):

For alkaline phosphatase (ALP) determination, ALP hydrolyzes p-nitrophenylphosphate to yield p-nitrophenol and inorganic phosphate (Pi) in an alkaline pH solution. The amount of p-nitrophenol formed is directly proportional to the activity of ALP enzymes in the serum samples.



2.5.10 Serum insulin determination and calculation of HOMA-β and HOMA-IR scores

The concentrations of serum insulin were measured by an enzyme-linked immunosorbent assay (ELISA) method using an ultrasensitive rat insulin ELISA kit in a multi-plate ELISA reader. The serum lipid profile (total cholesterol, HDL- and LDL cholesterol, and triglycerides), fructosamine, urea and creatinine concentrations as well as liver function enzymes were measured with an Automated Chemistry Analyzer. Computation of the homeostatic model assessment (HOMA-IR and HOMA-β) using were conducted using serum insulin and FBG concentrations measured at the end of the experimental period using the formula below:

$$\text{HOMA-IR} = [\text{Insulin (U/l)} \times \text{Blood glucose (mmol/L)}] / 22.5$$

$$\text{HOMA-}\beta = [20 \times \text{Insulin (U/l)}] / [\text{Blood glucose (mmol/L)} - 3.5]$$

Conversion factor: Insulin (1 U/l = 7.174 pmol/L)

Blood glucose (1 mmol/l = 18 mg/dL)

2.5.11 Liver glycogen determination

2.5.11.1 Preparation of reagents and standards

- *30% KOH saturated with Na₂SO₄ solution:* A 30 g of KOH (M.W. of 56.11 g/mol) was dissolved in 100 ml of distilled water and Na₂SO₄ (M.W. of 142.04 g/mol) was added until it was unable to be saturated.
- *5% phenol solution:* A 5 g of phenol (M.W. of 94.11 g/mol) was dissolved in 100 ml distilled water.
- *Glycogen stock solution:* A 5 mg of glycogen was dissolved in 5 ml distilled water (1 mg/ml).
- *Glycogen standards:* 5, 10, 20, 40, 80, 160, 320, and 640 µL of the glycogen stock solution were each diluted in 995, 990, 980, 960, 920, 840, 680, and 360 µL of distilled water respectively thus giving concentrations of 5, 10, 20, 40, 80, 160, 320, and 640 µg/mL respectively.

2.5.11.2 Method

The liver glycogen levels were measured photometrically using the phenol-sulphuric acid method as described by Lo *et al.*, (1970). Liver samples (< 1.0 g) were weighed, placed in the bottom of Eppendorf tubes with forceps and then placed on ice. The samples were immersed with 1.5 ml of 30% potassium hydroxide (KOH) saturated with sodium sulphate (Na₂SO₄) and placed in a boiling water bath for 30 minutes or more for digestion of liver tissue. The samples were then removed from the water bath and cooled on ice. Once cooled, 2 ml of 95% ethanol was added and placed on ice for a further 30 minutes. The digested liver samples were then centrifuged at 840 x g (2812 rpm) for 30 minutes. The resulting supernatant was aspirated, and the glycogen precipitate was dissolved in 3.0 mL of distilled water. A 100 µL of this solution was transferred into a clean test tube containing 900 µL of distilled water. Glycogen standards at concentrations of 5, 10, 20, 40, 80, 160, 320 and 640 µg/mL were prepared from a stock solution of 1 mg/mL glycogen (Oyster, Type II, Sigma-Aldrich, USA). The samples and standards were prepared in duplicates. Once the samples and standards were prepared, 1 ml of 5% phenol was added followed by the rapid addition of 5 ml 96-98% sulphuric acid (H₂SO₄). The samples and standards were left to stand for 10 minutes at room temperature and the absorbance was then read at 490 nm using a spectrophotometer. Distilled water was used as a blank. The liver glycogen concentration was calculated from the glycogen standard curve.

2.5.12 Assay of specific antioxidant enzymes and lipid peroxidation

➤ Preparation of homogenization buffer:

A 50 mM sodium phosphate buffer with triton X-100 at pH 7.4 was prepared as follows:

2.998 g of monobasic sodium phosphate (M.w., 119.98 g/mol) was dissolved in distilled water (dH₂O) and the volume brought up to 500 mL in a volumetric flask to yield a 500 mL of a 50 mM mono-basic sodium phosphate solution. A 3.525 g of dibasic sodium phosphate (M.w., 141.56 g/mol) was dissolved in dH₂O and the final volume made up to 500 mL in a volumetric flask to yield a 500 mL of 50 mM dibasic sodium phosphate solution. While stirring, the prepared 50 mM mono-basic sodium solution was added, steadily, to the prepared 50 mM dibasic sodium phosphate solution, until a pH of 7.4 was attained. A 0.5 % (v/v) triton X-100 was added to the above mixture and the pH was again adjusted to 7.4 with 50 mM mono-basic sodium phosphate solution. The buffer was then stored at 4°C until further use.

➤ Preparation of sample homogenates

0.5 g of each organ was weighed and finely minced for better homogenization. The finely minced tissues were homogenized with an electronic homogenizer in 5 mL ice cold homogenization buffer (pH 7.4). The mixture was then transferred into 2 mL microcentrifuge tubes and centrifuged for 15 min at 15000 rpm using a refrigerated microcentrifuge set at 4°C.

2.5.12.1 Determination of thiobarbituric acid reactive substance (TBARS) concentration as malondialdehyde (MDA) equivalent to measure level of lipid peroxidation

This assay was aimed at determining the level of lipid peroxidation reducing activity of caffeine at different concentrations. It is based on the reaction between a thiobarbituric acid reactive substance (TBARS) such as malondialdehyde (MDA) and thiobarbituric acid. When incubated at 95°C, a red, fluorescent 1:2, MDA: TBA adduct forms which can be spectrophotometrically measured at an absorbance of 532 nm (Mayne, 2003).

Preparation of reagents

➤ 25 mL of 8.1% sodium dodecyl sulfate (SDS) solution

2.025 g of SDS (M.w., 288.38 g/mol) was dissolved in MiliQ water and the final volume was made up to 25 mL with MiliQ water in a volumetric flask.

➤ 100 mL of 20% acetic acid solution (pH 3.5)

20 mL of glacial acetic acid was made up to 100 mL with MiliQ water and a sodium hydroxide solution was used to adjust the pH of the solution to 3.5.

➤ 250 mL of 0.25% TBA solution

0.625 g of TBA (M.w., 144.14 g/mol) was dissolved in MiliQ water and the final volume was made up to 250 mL with MiliQ water in a volumetric flask.

Procedure

Thiobarbituric acid reactive substances, expressed as MDA concentration were measured to determine the extent of lipid peroxidation using the protocol described by Fraga *et al.*, (1957). A 200 μ L of the sample or MDA standards were thoroughly mixed with 200 μ L of 8.1% SDS solution, 750 μ L of 20% acetic acid, 2 mL of 0.25% TBA and 850 μ L of distilled water. Subsequently, the resulting mixture was heated in a water bath at 95°C for 1 hour and allowed to cool to room temperature. The absorbance of the resulting solution was then read at 532 nm and the concentration of TBARS in the samples was calculated from the MDA standard curve.

Calculation of MDA concentrations of samples

A standard curve was obtained by plotting the absorbance read at 532 nm of the MDA standards against their corresponding concentrations using Microsoft Excel, 2016. From this standard curve, the MDA concentration of each sample was extrapolated from their respective absorbance at 532 nm.

2.5.12.2 Determination of reduced glutathione (GSH) level

The principle of this assay involves the oxidation of GSH by 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) to 5'-thio-2-nitrobenzoic acid (TNB), which is a yellow derivative that is measurable at 415 nm. The formed GSSG can be recycled to GSH by glutathione reductase in the presence of NADPH (Rahman *et al.*, 2006).

Preparation of reagents

➤ 50 mL of 0.2 M sodium phosphate buffer (pH 7.8)

A 50 mL of 0.2 M mono-basic sodium phosphate solution was prepared by dissolving a 1.199 g of mono-basic sodium phosphate (M.w., 119.9 g/mol) in distilled water and the final volume was made up to 50 mL with distilled water. A 100 mL of 0.2 M dibasic sodium phosphate solution was prepared by dissolving a 2.831 g of dibasic sodium phosphate (M.w., 141.56

g/mol) in distilled water and the final volume was made up to 100 mL with distilled water. 0.2 M mono-basic sodium phosphate solution was steadily added to the 0.2 M dibasic sodium phosphate solution, while stirring until a pH of 7.8 was reached. This was then stored at 4 °C until further use.

➤ 20 mL of 10% TCA solution

2 g of TCA (M.w., 163.4 g/mol) was dissolved in MiliQ water and the final volume was brought up to 20 mL with MiliQ water.

➤ 50 mL of 0.5 mM DTNB solution

0.01 g of DTNB (M.w., 396.35 g/mol) was dissolved in MiliQ water and the final volume was brought up to 50 mL with MiliQ water in a volumetric flask.

Assay procedure

Reduced glutathione concentration was measured in tissue samples. All samples were first precipitated with 10% TCA (300 μ L TCA was added to 300 μ L of each sample) and then centrifuged at 2000 rpm for 10 min at room temperature (25 °C).

Into a 96-well plate, the following reaction mixture, containing 80 μ L of supernatant, 40 μ L of 0.5 mM DTNB and 200 μ L of 0.2 M sodium phosphate buffer (pH 7.8), was added. The absorbance of this reaction mixture was then measured at 415 nm after 15 min incubation at 25°C.

Calculation of GSH concentration of samples

A standard curve was obtained by plotting the absorbance read at 415 nm of the GSH standards against their related concentrations using Microsoft Excel, 2016. From this standard curve, the GSH concentration of each sample was extrapolated from their respective absorbance at 415 nm.

2.5.12.3 Determination of catalase (CAT) activity

Principle

Catalase activity was determined according to the method of Sinha A., 1971. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 , with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570-610nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Preparation of reagents

➤ **5% $\text{K}_2\text{Cr}_2\text{O}_7$ (Dichromate Solution)**

5g of $\text{K}_2\text{Cr}_2\text{O}_7$ (Hopkins & Williams, England) was dissolved in 80mL of distilled water and made up to 100mL with same.

➤ **0.2M H_2O_2 (Hydrogen peroxide)**

11.50 mL of 30% (w/w) H_2O_2 was diluted with distilled water in a volumetric flask and the solution made up to 500 mL.

➤ **Dichromate/acetic acid**

This reagent was prepared by mixing 5% solution of $\text{K}_2\text{Cr}_2\text{O}_7$ with glacial acetic acid (1:3 by volume) and could be used indefinitely.

➤ **Phosphate buffer (0.01M, pH 7.0)**

3.58g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 1.19g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ dissolved in 900mL of distilled water. The pH adjusted to 7.0 and distilled water added to make up to 1 litre.

Assay procedure

Colorimetric determination of H_2O_2 : Different amounts of H_2O_2 , ranging from 10 to 100 μmoles were taken in small test tubes and 2mL of dichromate/acetic acid was added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature,

the volume of the reaction mixture was made to 3ml and the optical density measured with a spectrophotometer at 570nm. The concentrations of the standard were plotted against absorbance.

Calculation of catalase activity of samples

Catalase activity was calculated by plotting the standard curve and the concentration of the remaining H₂O₂ was extrapolated from the curve.

H₂O₂ consumed = 800μmoles – H₂O₂ remaining

Catalase activity = H₂O₂ consumed /mg protein

2.5.13 Histopathological examination of pancreatic tissues

Immediately after the collection of the pancreas, a small section was cut and placed in a 1 mL eppendorf tube containing 10% neutral buffered formalin solution. Formalin was changed weekly until all tissues were processed. For tissue processing, the section of each organ collected was dehydrated in 2 changes of 70% Ethanol (EtOH) of 1 hour each, followed by 1 change of 80% EtOH for 1 hr, 1 change of 90% EtOH for 1 hr and 1 change of 100% EtOH overnight. After which the tissues were cleared in 3 changes of xylene for 1.5 hrs each. Tissue sections were then embedded in paraffin wax. The paraffin blocks were trimmed to the appropriate size. Sections were cut at 3 μm and placed in a 40-45°C water bath. Thereafter, sections were mounted onto slides and allowed to air-dry overnight. Following air drying, the slides were deparaffinised in 2 changes of xylene for 5 and 2 min respectively. Slides were then rehydrated in 1 change of 100% EtOH for 2 min, 1 change of 90% EtOH for 2 min, 1 change of 70% EtOH for 2 min, 1 change of 50% EtOH for 2 min, and then placed in d.H₂O. Slides were stained with haematoxylin as the primary stain for 5 min and washed under running water. Slides were then counterstained with eosin for 3 min. The slides were dipped in 90-100% EtOH followed by xylene, wiped and cover slipped using DPX mounting agent cover-slipped and viewed with Leica slide scanner (SCN 4000, Leica Bio-systems, Germany).

2.6 Statistical analysis

All data are presented as mean ± SD. Data were analysed using a statistical software package (SPSS for windows, USA), by using one-way ANOVA and Tukey's HSD post hoc test.

Values were considered significantly different at $p < 0.05$

CHAPTER 3

RESULTS

3.1 *In vitro* Studies

3.1.1 DPPH- 1,1-diphenyl-2-picrylhydrazyl Radical Scavenging Activity

The DPPH radical scavenging activities of caffeine are presented in **Figure 3.1**. Caffeine demonstrated powerful free radical scavenging activity which were similar with most other standard antioxidants used in this experiment.

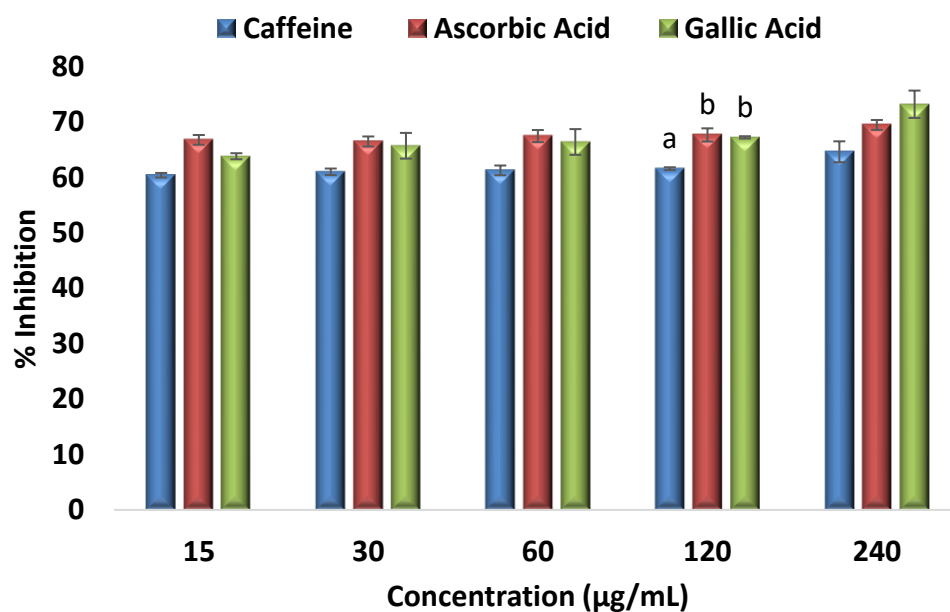


Figure 3.1: DPPH radical scavenging activity of caffeine and standard drugs. Data are presented as mean \pm SD. ^{ab}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.1.2 Ferric reducing antioxidant power (FRAP) assay

The total reducing power (in terms of percentage gallic acid equivalent) of caffeine was compared to ascorbic acid and presented in **Figure 3.2**. According to the results, the total reducing power of caffeine was found to increase steadily with increasing concentrations of the samples. However, caffeine demonstrated significantly ($p < 0.05$) lower Fe^{3+} to Fe^{2+} reductive ability than the standard compound used (Ascorbic acid) in this experiment.

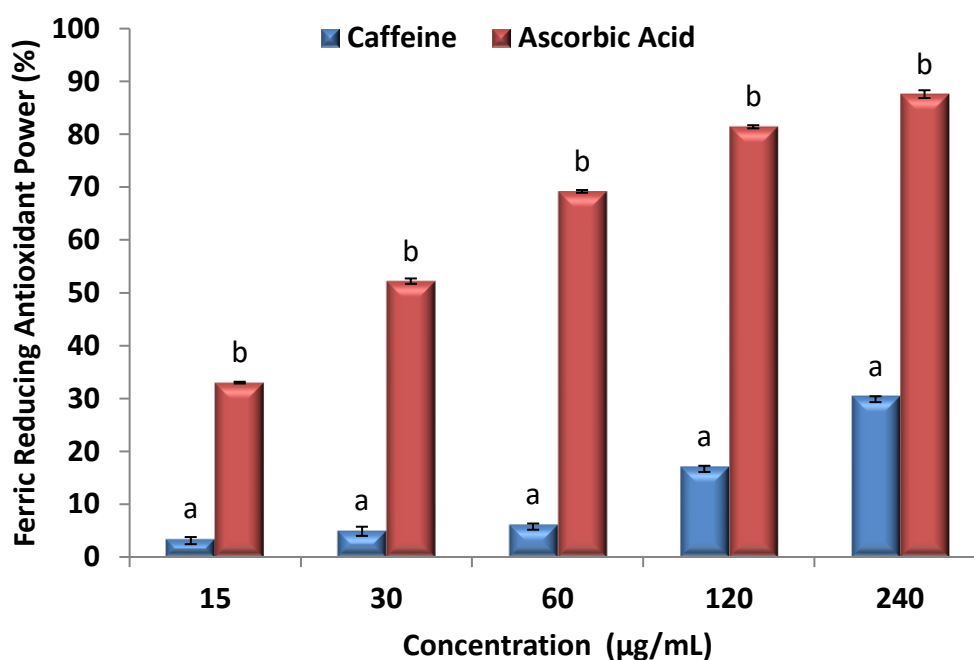


Figure 3.2: Ferric reducing antioxidant power of the different concentrations of caffeine and ascorbic acid (Standard). Data are presented as mean \pm SD. ^{ab}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.1.3 Nitric Oxide (NO) Scavenging Activity

Although a similar NO scavenging effects was observed for caffeine with other standard compounds at lower concentrations (15 and 30 $\mu\text{g/mL}$), standard compounds has a higher NO scavenging activity compared to caffeine for higher concentrations (**Figure 3.3**). Hence, caffeine may not have strong nitric oxide scavenging effects as found for ascorbic acid and Trolox, particularly in higher concentration (**Figure 3.3**).

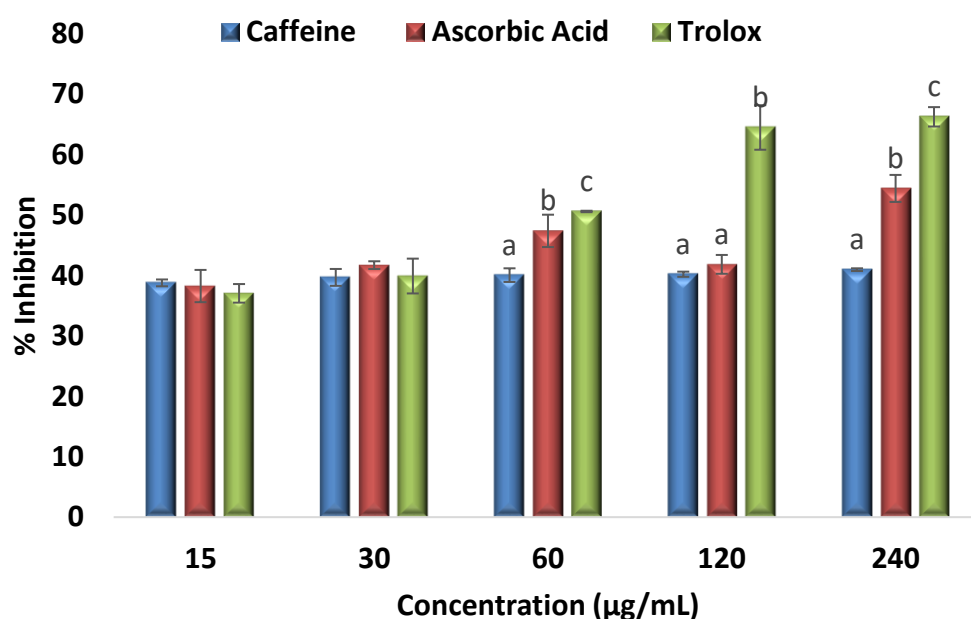


Figure 3.3: Nitric oxide (NO) scavenging activity of caffeine and standard compounds. Data are presented as mean \pm SD. ^{abc}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.1.4 Hydroxyl radical scavenging activity (Deoxy-2- ribose assay)

The results of the hydroxyl radical scavenging (HRS) assay showed that caffeine could scavenge hydroxyl radicals produced by Fenton's reaction in a non-dose dependent manner (**Figure 3.4**). Although highest HRS activity was observed for quercetin at 240 $\mu\text{g/mL}$ concentration and dose-dependent effects were observed for gallic acid, no such effects were observed for caffeine. The HRS activities of caffeine and gallic acid were relatively lower ($<30\%$) even at higher concentration (240 $\mu\text{g/mL}$) used in this study.

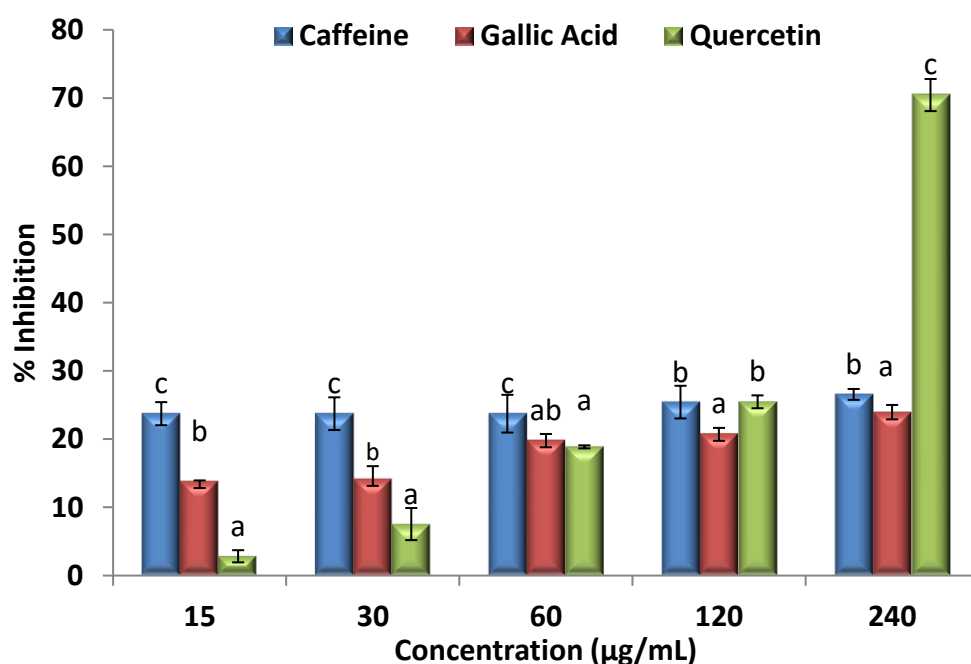


Figure 3.4: Deoxy-2- ribose hydroxyl radical scavenging (HRS) activity of caffeine. Data are presented as mean \pm SD. ^{abc}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.1.5 Measurement of *in vitro* inhibition of alpha amylase activity

The alpha amylase and alpha glucosidase inhibitory activities of the caffeine and standard drug are shown in **Figure 3.5. and 3.6**. Although caffeine inhibited alpha amylase and alpha glucosidase activities in a non-dose dependent manner, the results were not significantly different among the concentrations. However, it demonstrated significantly lower ($p < 0.05$) α -amylase α -glucosidase inhibitory activities compared to acarbose in all concentrations used in this experiment.

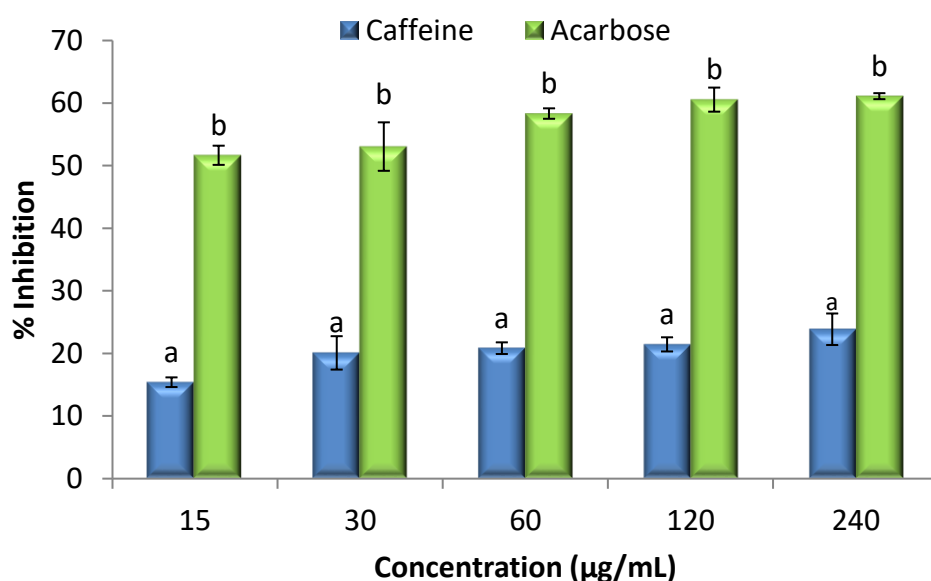


Figure 3.5: The effect of caffeine on the activities of alpha amylase enzyme *in vitro*. Data are presented as mean \pm SD. ^{ab}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.1.6 Measurement of *in vitro* inhibition of alpha glucosidase activity

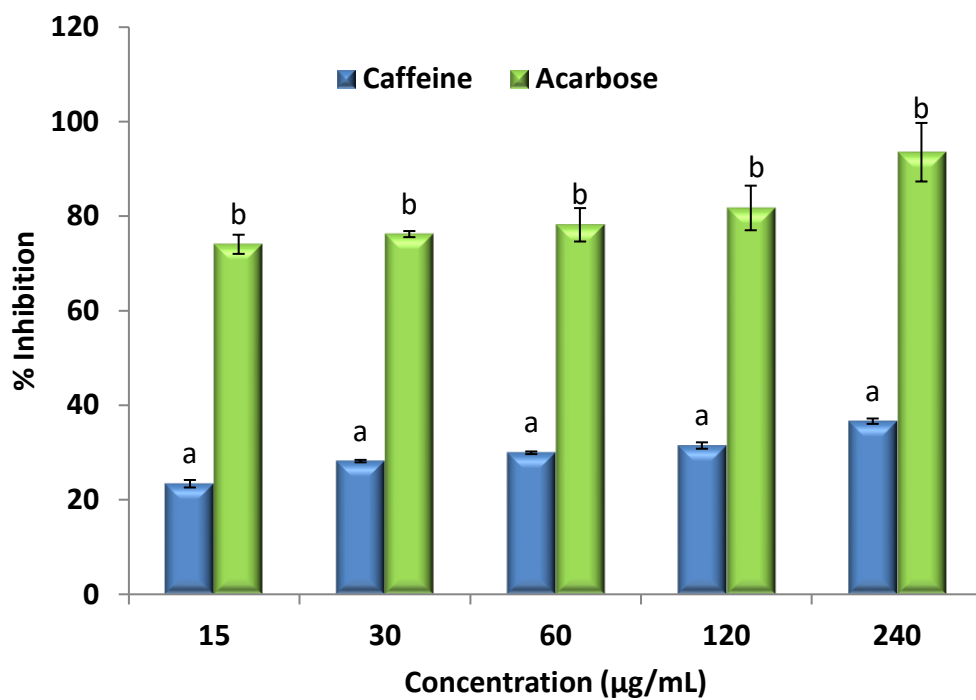


Figure 3.6: Effects of caffeine on alpha glucosidase enzyme activity *in vitro*. Data are presented as mean \pm SD. ^{ab}Values with different letter over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.1.7 *In vitro* pancreatic lipase inhibitory activity assay

Although caffeine demonstrated dose-dependent anti-lipase activity, the level of inhibitory activities was very low at different concentrations used. The pancreatic lipase inhibitory activity of caffeine was also significantly lower than the standard drug (orlistat) used in this assay.

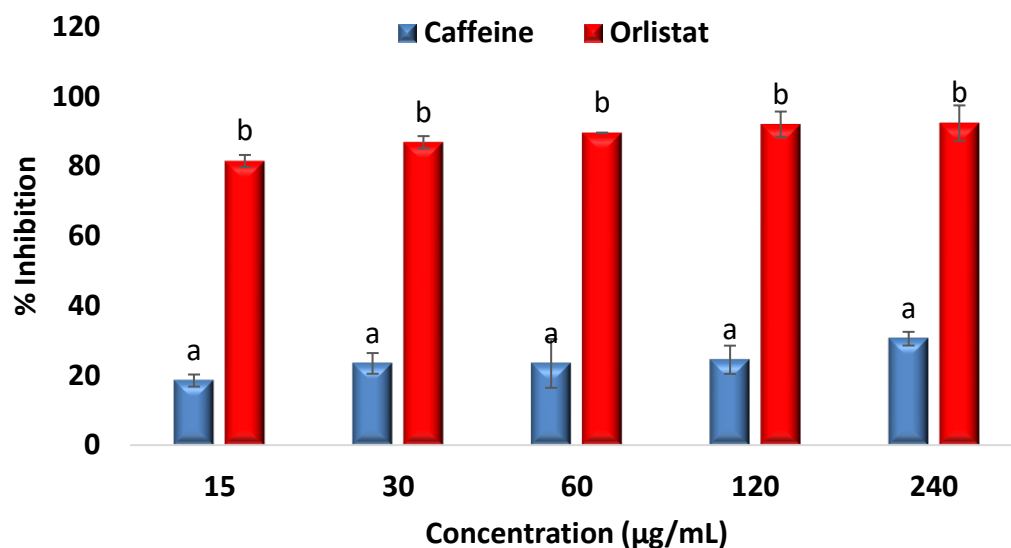


Figure 3.7: Effect of caffeine on the activity of lipase enzyme *in vitro*. Data are presented as mean \pm SD. ^{ab}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.2 *Ex vivo* Studies

3.2.1 The effect of caffeine on lipid peroxidation in pancreas

The effects of caffeine on pancreatic lipid peroxidation was measured as MDA. Pancreatic MDA levels were significantly ($p < 0.05$) increased after the incubation pancreatic tissue with FeSO_4 , representing the induction of lipid peroxidation. Incubation with caffeine led to a dose-dependent reduction of lipid peroxidation, demonstrating its anti-peroxidative effect ($p < 0.05$).

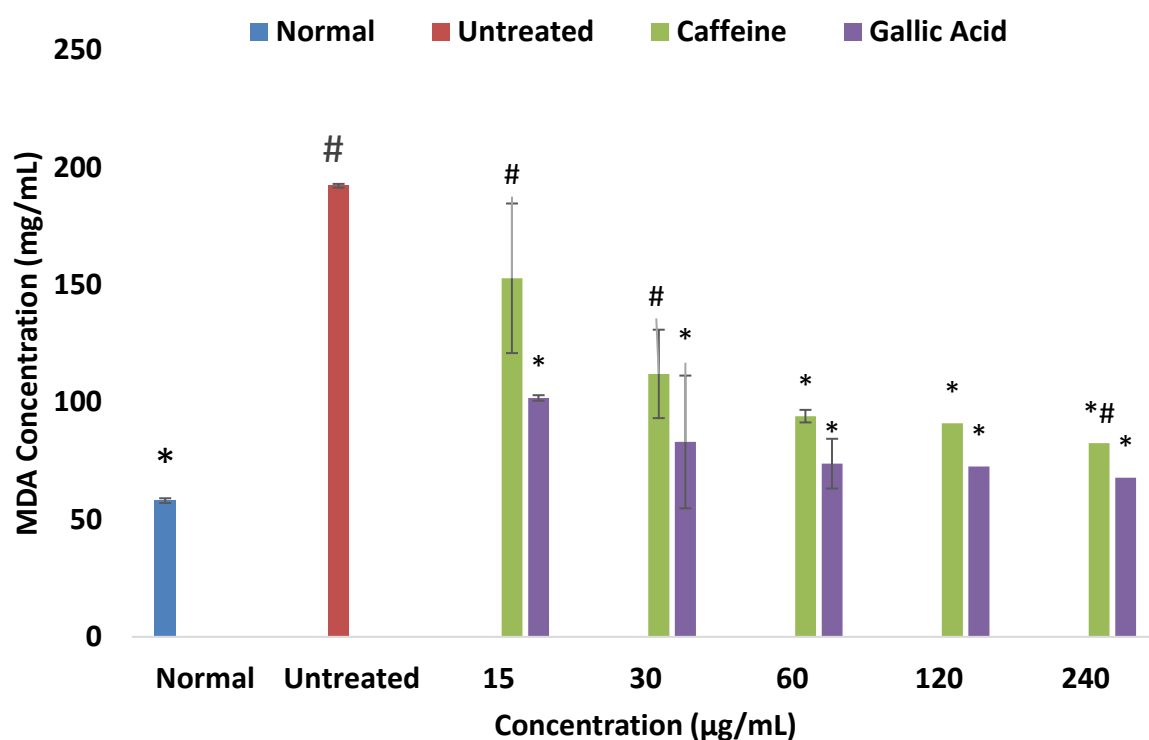


Figure 3.8: The effect of caffeine on lipid peroxidation in pancreas. Data are presented as mean \pm SD. *Significantly different from untreated control and #Significantly different from normal (Tukey's-HSD multiple range post hoc test, $p < 0.05$). Normal = tissues not incubated with FeSO_4 or compound; Untreated = tissues incubated with FeSO_4 only.

3.2.2 The effect of caffeine on GSH activity in oxidative pancreatic injury.

The GSH level was significantly ($p < 0.05$) depleted after the incubation of pancreatic tissue with FeSO_4 indicating the induction of oxidative stress. However, the GSH activity was not significantly improved either with caffeine or standard compound (gallic acid) used in this study at different concentrations.

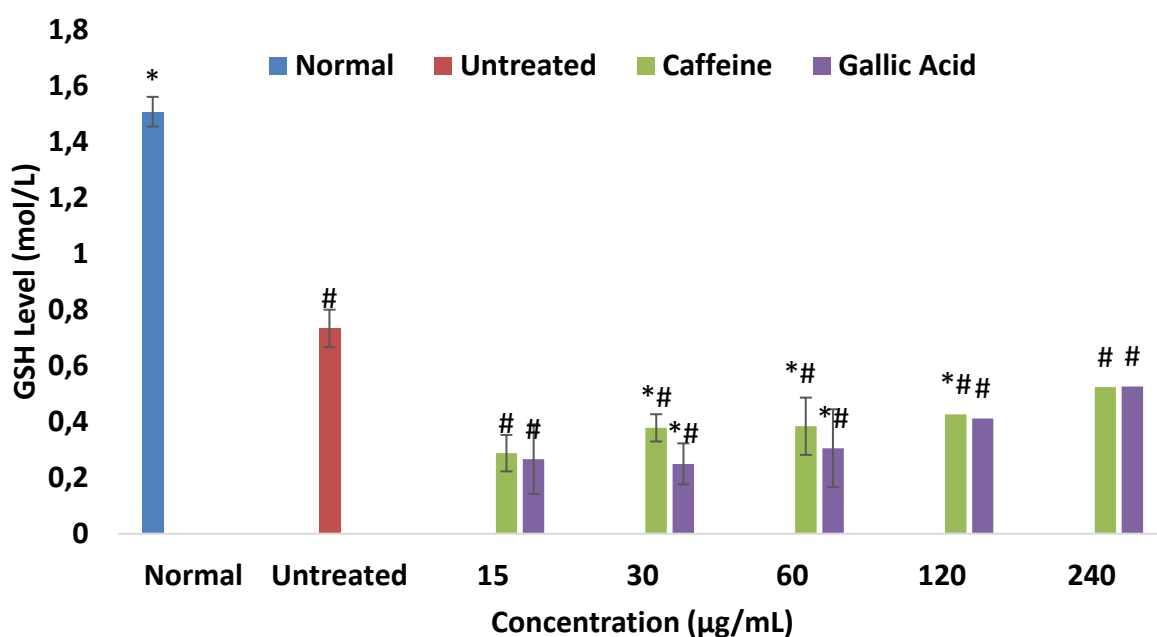


Figure 3.9: The effect of caffeine on GSH level in oxidative pancreatic injury. Data are presented as mean \pm SD. *Significantly different from untreated control, #Significantly different from normal and ^{a,b}Different alphabets over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $P < 0.05$). Normal = tissues not incubated with FeSO_4 or compound; Untreated = tissues incubated with FeSO_4 only.

3.2.3 The effect of caffeine on SOD activity in oxidative pancreatic injury

Incubation of pancreatic tissue with FeSO_4 led to the reduction of SOD activity as depicted in **Figure 3.10**, further confirming the induction oxidative imbalance. These were significantly ($p < 0.05$) reversed with increasing concentration of caffeine, indicating an antioxidative activity of caffeine.

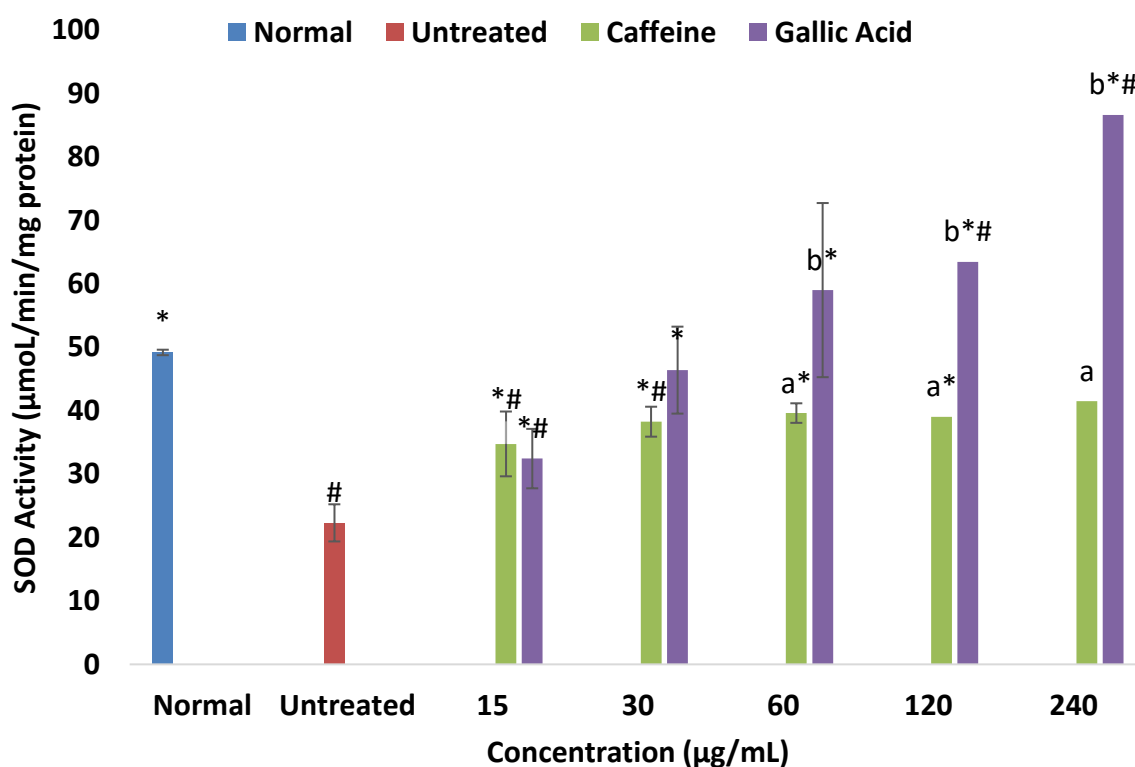


Figure 3.10: The effect of caffeine on SOD activity in oxidative pancreatic injury. Data are presented as mean \pm SD. *Significantly different from untreated control, #Significantly different from normal and ^{a,b}Different alphabets over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$). Normal = tissues not incubated with FeSO_4 or compound; Untreated = tissues incubated with FeSO_4 only. SOD, Superoxide dismutase.

3.2.4. Effects of caffeine on catalase activity in oxidative pancreatic injury.

Incubation pancreatic tissue with FeSO_4 led to decrease in catalase activity as depicted by an increase in H_2O_2 levels. These were significantly ($p < 0.05$) reversed with increasing concentration of caffeine, indicating its antioxidative activity in a non-dose dependent manner.

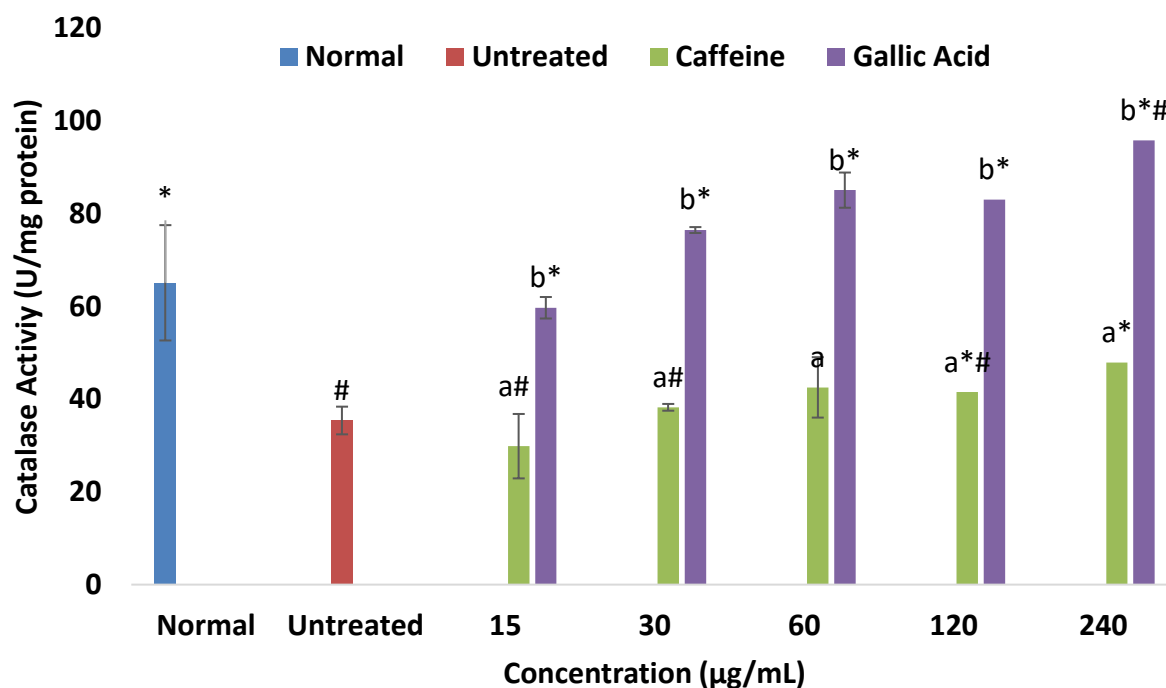


Figure 3.11: The effect of caffeine on catalase activity in oxidative pancreatic injury. *Significantly different from untreated control, #Significantly different from normal, and ^{a,b}Different letters above the bars for a given concentrations are significantly different from each other (Tukey's-HSD multiple range post hoc test, $P < 0.05$). Normal = tissues not incubated with FeSO_4 or compound; Untreated = tissues incubated with FeSO_4 only. Data are presented as mean \pm SD.

3.2.5. The effects of caffeine on glucose absorption in isolated rat jejunum

The result showed that the amount of glucose absorbed by isolated rat jejunum in the presence of caffeine was reduced significantly in all concentrations of caffeine used in this experiment (**Figure 3.12**). These results show that caffeine has significant effects on inhibiting small intestinal glucose absorption.

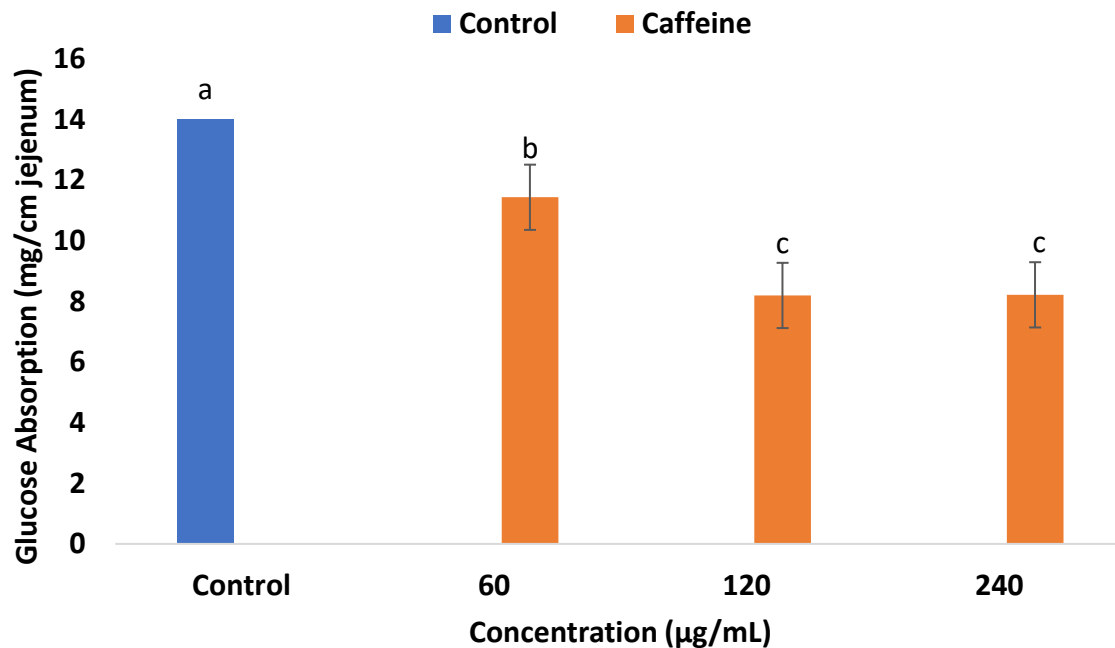


Figure 3.12: The effects of caffeine on glucose absorption in isolated rat jejunum. Data are presented as mean \pm SD. ^{abc}Values with different letters over the bars are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.2.6. The effects of caffeine on glucose uptake in rat psoas muscle

The data shows that the amount of glucose uptake by psoas muscle in the presence of caffeine increased significantly only at concentration 240 $\mu\text{g/mL}$ compared to the control and other lower concentrations (60 and 120 $\mu\text{g/mL}$) used in this experiment (**Figure 3.13**). This means that caffeine has muscle glucose uptake enhancing activity only at higher concentrations.

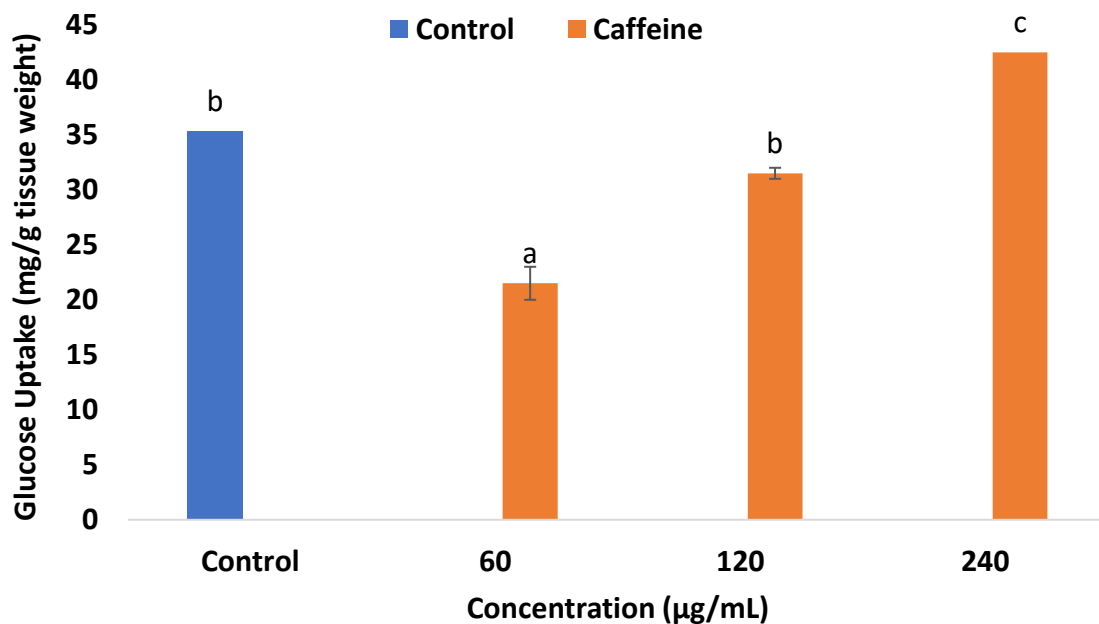


Figure 3.13: The effects of caffeine on glucose uptake in rat psoas muscle. Data are presented as mean \pm SD. ^{abc}Values with different letters over the bars for are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

Table 3.1: IC₅₀ values of biological activities of caffeine and standard drugs

Biological Activities	Caffeine	Ascorbic Acid	Gallic Acid	Trolox	Quercetin	Acarbose	Orlistat
	(µg/mL)						
DPPH	7×10 ⁻³	5×10 ⁻⁷	0.163	-	-	-	-
FRAP	>1000	28.76	-	-	-	-	-
NO	>1000	185.3	-	52.21	-	-	-
HRS	1.49×10 ¹²	-	>1000	-	185.67	-	-
α-amylase	>1000	-	-	-	-	9.68	-
α-glucosidase	>1000	-	-	-	-	0.5	-
Pancreatic lipase	2.18×10 ¹³	-	-	-	-	-	4.89×10 ⁻¹²

Data are presented as mean; n =3; Lower IC₅₀ value = better activity

DPPH-1,1-diphenyl-2-picrylhydrazyl radical, FRAP- Ferric reducing antioxidant power, NO- nitric oxide radical, HRS- Hydroxyl radical scavenging activity.

Table 3.2: IC₅₀ values of biological activities of caffeine and standard drugs

Biological Activities	Caffeine (µg/mL)	Gallic acid (µg/mL)
Catalase	347.84	4.28
GSH	-	-
SOD	64.14	39.45
Lipid peroxidation	678.21	833.61
Glucose absorption	0.0000029	-
Glucose uptake	398.13	-

Data are presented as mean; n = 3. GSH- Reduced glutathione, SOD- Superoxide dismutase.

3.3. *In vivo* Studies

3.3.1. Food and fluid intake

Figure 3.14, shows the mean food (g/rat/day) and fluid (mL/rat/day) intake in different animal groups over the 7-week experimental period. Food and fluid intake of diabetic experimental groups were significantly higher ($p < 0.05$) compared to the NC group when DBC group showed significantly lower fluid intake compared to most other treated with caffeine (except DCAFM) or standard anti-diabetic drugs (**Figure 3.14**).

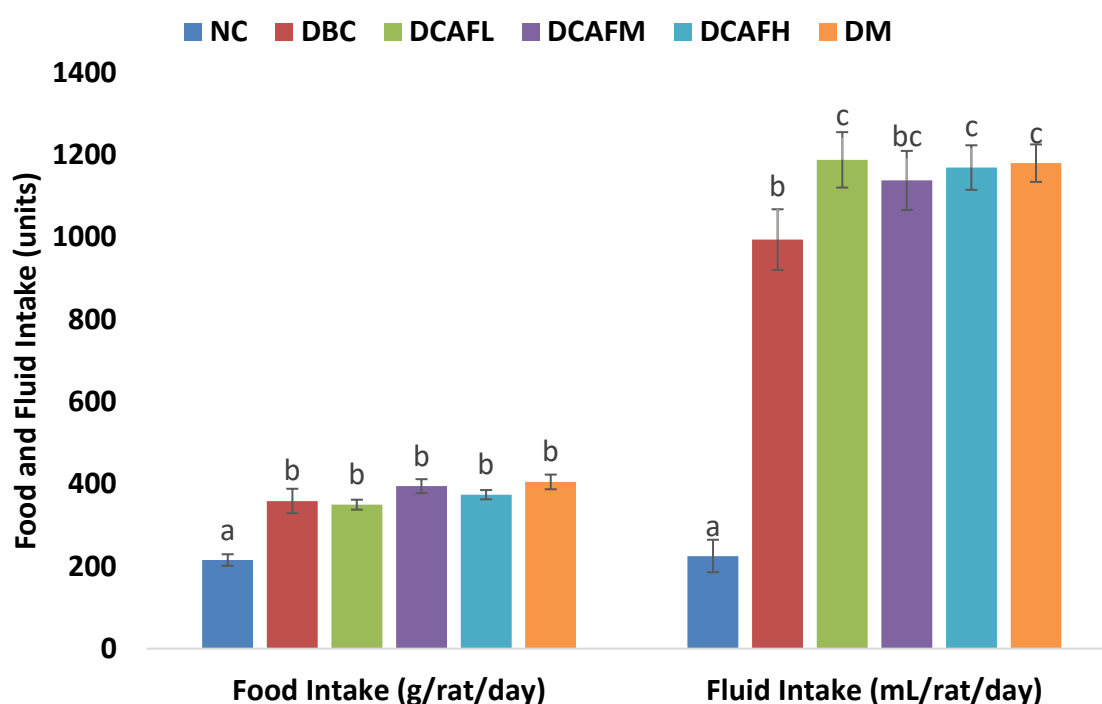


Figure 3.14: The effects of oral treatment of caffeine on food and fluid intake in the different group of rats. The results are expressed as mean \pm SD of 7 rats. ^{abc}Different alphabets over the bars for a given parameter indicate significance of difference (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.3.2. Body weight (BW) change

Figure 3.15 shows the weekly BW change over the 7-week experimental period. All the animal groups had a similar mean initial BW at the beginning of the study however as soon as STZ was injected, the BW of all diabetic groups were significantly lower ($p < 0.05$) compared to the NC group throughout the experimental period. However, although there were no significant differences in BW observed between the DBC and the treated groups during the early weeks, the BW of animals treated with the high dose of caffeine (DCAFH) and metformin (DM) was significantly higher than the other diabetic groups (DBC, DCAFL, DCAFM) when the induction of BW for DM group was significantly higher than the DCAFH group (**Figure 3.15**).

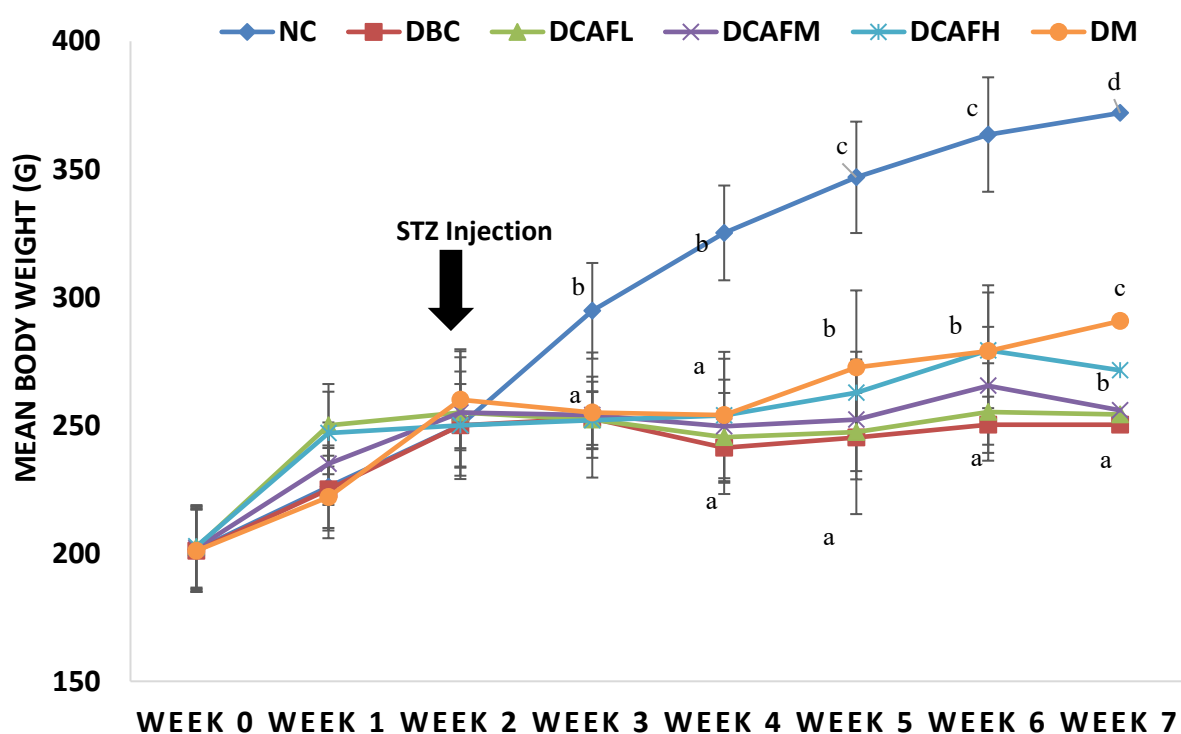


Figure 3.15: The effects of oral treatment of caffeine on the mean body weight change in the different group of rats during the experimental period. The results are expressed as mean \pm SD of seven rats. ^{ab}Different alphabets near the lines for a given week indicate significance of difference (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.3.3. Weekly blood glucose

Figure 3.16 shows the level of weekly blood glucose (BG) of different animal groups during the 5-week experimental period. The BG of the DBC and treatment groups were significantly higher ($p < 0.05$) compared to the NC group, however, the BG levels of DCAFL and DM was significantly lower ($p < 0.05$) compared to the other diabetic groups (DBC, DCAFL and DCAFM) (**Fig. 3.16**). Blood glucose was gradually decreased with the period of treatment with the high dose of caffeine (DCAFH) which was comparable to the effect of standard diabetic drug (DM).

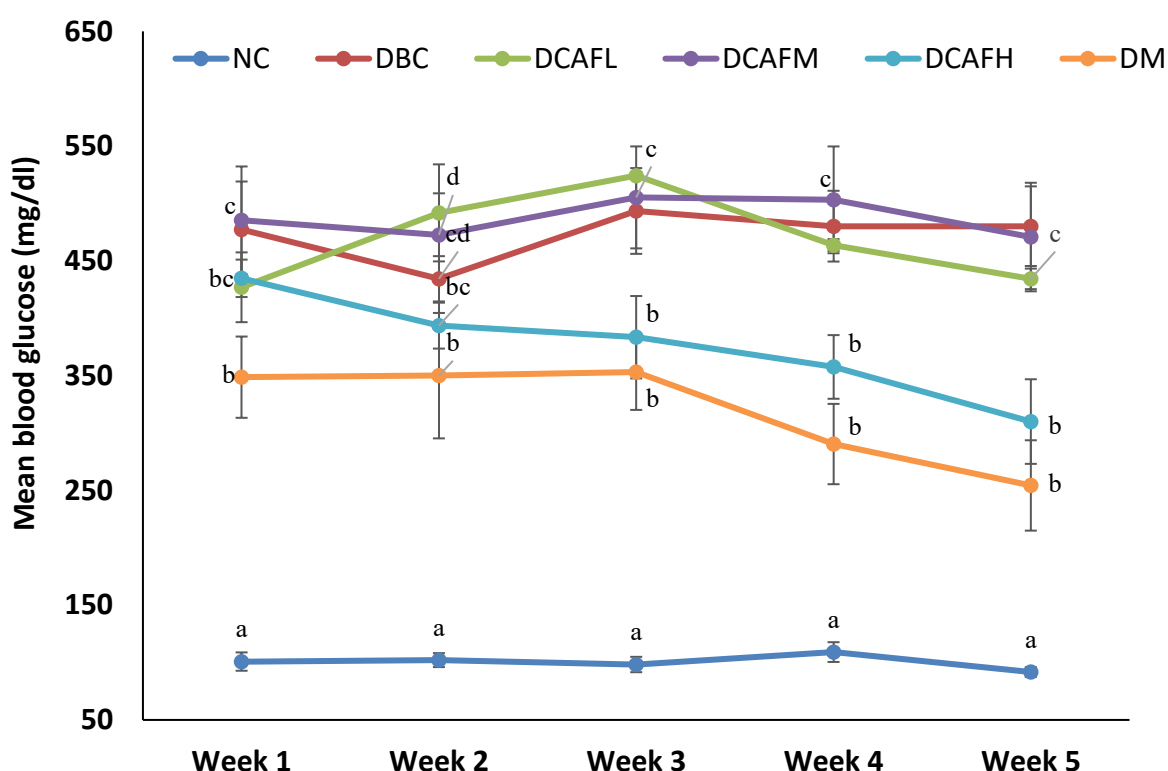


Figure 3.16: The effects of caffeine treatment on weekly blood glucose concentrations in different group of rats. The results are expressed as mean \pm SD seven rats. ^{a-d}Different alphabets near the lines for a given week indicate significance of difference (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.3.4. Oral glucose tolerance test (OGTT)

The data for OGTT are presented in **Figure 3.17**. The BG levels of the DCAFH group after 30 and 120 min and the BG of DM group after 90 and 120 min were significantly lower ($p < 0.05$) than the DBC group. No significant differences were observed between the other groups at different time points.

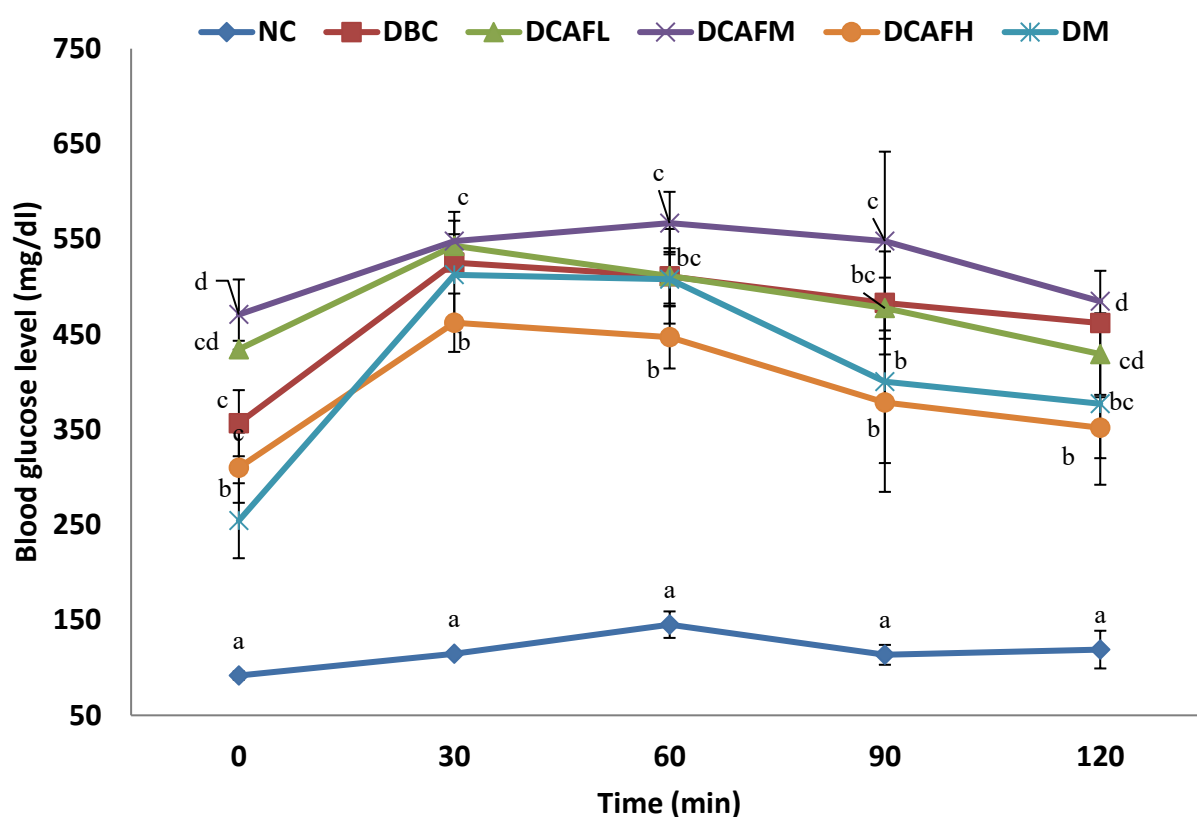


Figure 3.17: Oral glucose tolerance test (OGTT) for all groups of animals in the last week of experimental period. The results are expressed as mean \pm SD of 7 rats. ^{a-d}Different alphabets near the lines for a given time indicate the significance of difference (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

Table 3.3: Area under the curve (AUC) values for the OGTT activity

Area under curve (AUC)	NC	DBC	DCAFL	DCAFM	DCAFH	DM
	478.58	1928.71	1963.73	2140.63	1618.75	1736.67

$$AUC = [(B2 + B1) / 2] \times (A2 - A1)$$

B1 and B2 = Initial and final blood glucose values (mg/dl) at a given time respectively

A1 and A2 = Initial and final time periods (min) respectively

3.3.5. Serum lipid profile

Figure 3.18 shows the data for the serum lipid profile in different experimental groups. There was no significant difference in the level of serum triglycerides and LDL-cholesterol between the groups. However, the total cholesterol of DCAFL and DCAFH groups were significantly lower than the DC group. Additionally, HDL-cholesterol of DCAFH group was significantly higher than the DC, DACFL and DCAFM groups when no significant difference was observed among the other groups.

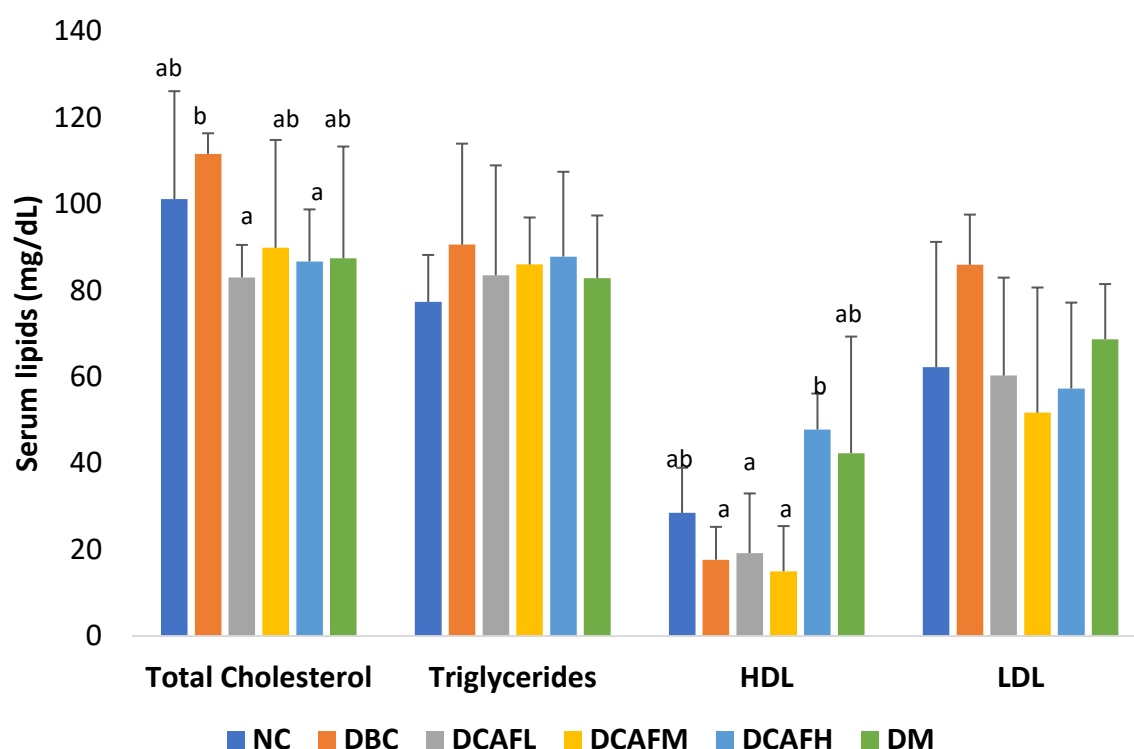


Figure 3.18: Serum lipid profile in different animal groups at the end of the experimental period. The results are expressed as mean \pm SD of 5-7 rats. HDL: high density lipoproteins; LDL: low density lipoproteins.

3.3.6. Liver weights and liver glycogen

The data for liver weight, relative liver weight and liver glycogen levels are presented in **Table 3.4**. The NC, DCAFH and DM group had a significantly higher liver weight compared to all other groups, while relative liver weight of DCAFH group was significantly higher than the DBC group. Similarly, the liver glycogen DCAFH group was also significantly higher than the DBC group (**Table 3.4**). Overall the level of liver weight, relative liver weight and liver glycogen levels of DCAFH group were significantly higher than the DBC group when the results for DCAFH group were comparable to NC group.

Table 3.4: Liver weights and liver glycogen levels in different animal groups at the end of the experimental period.

Rat group	NC	DBC	DCAFL	DCAFM	DCAFH	DM
Liver weight (g)	13.72±0.10 ^c	9.83±0.29 ^a	10.31±0.21 ^a	10.01±0.68 ^a	12.02±0.47 ^b	13.27±0.83 ^{bc}
*Relative liver weight (%)	3.69±0.03 ^a	3.79±0.11 ^a	3.89±0.08 ^{ab}	3.92±0.27 ^{ab}	4.43±0.17 ^{bc}	4.76±0.30 ^c
Liver glycogen (µg/g) tissue	5.69±0.99 ^c	2.27±0.20 ^{ab}	3.33±0.47 ^{ab}	3.30±0.35 ^a	4.87±0.60 ^c	4.57±0.95 ^{ab}

The results are expressed as mean ± SD of 5-7 rats. ^{a-c}Different alphabets within a row are significantly different from each other (Tukey's-HSD multiple range post hoc test, P<0.05)

*Relative liver weight (%) = [(liver weight in grams / body weight in grams)] x 100.

3.3.7. Serum insulin, fructosamine, HOMA-IR and HOMA-β

The level of serum insulin and pancreatic HOMA-β scores or pancreatic β-cell functions were significantly higher in the DCAFH group compared to the DBC group when the level of serum fructosamine and HOMA-IR scores were significantly lower in the DCAFH group compared to the DBC group (Table 3.5).

Table 3.5: Serum insulin, fructosamine, and HOMA-IR and HOMA-β scores in different animal groups at the end of the weeks experimental period.

Serum parameters/ Rat groups	NC	DBC	DCAFL	DCAFM	DCAFH	DM
Insulin (pmol/L)	70.78±12.46 ^c	9.53±3.20 ^a	14.79±3.20 ^a	18.11±6.48 ^{ab}	21.53±1.71 ^{ab}	44.57±0.72 ^b
Fructosamine(μmol/L)	569.71±69.89 ^a	676.2±34.09 ^c	683.20±25.71 ^c	679±33.13 ^c	637.67±47.80 ^{ab}	645.67±32.68 ^{ab}
HOMA-IR¹	1.17±0.18 ^a	3.90±0.16 ^d	2.21±0.5 ^b	2.93±0.2 ^c	2.29±0.8 ^b	2.23±0.12 ^b
HOMA-β²	123.54±1.05 ^d	1.63±0.09 ^a	1.99±0.14 ^a	2.23±0.27 ^a	4.37±0.22 ^b	11.69±0.53 ^c

The results are expressed as mean ± SD of 5-7 rats. ^{a-d}Different alphabets within a row for a given parameter are significantly different from each other (Tukey's-HSD multiple range post hoc test, P<0.05).

¹HOMA-IR = [Insulin (U/l) × Blood glucose (mmol/l)] / 22.5; ²HOMA-β = [20 × Insulin (U/l)] / [Blood glucose (mmol/l) – 3.5]. HOMA: homeostatic model assessment; IR: insulin resistance

3.3.8. Serum creatinine, Urea, AST and ALP

The data for serum creatinine, urea, AST and ALP are presented in **Table 3.6**. There was no significant difference in serum creatinine and AST levels, however, the ALP levels for the DCAFH and DM groups were significantly lower compared to all other diabetic groups which were also comparable to NC group. Results show that the serum urea levels of all diabetic groups were significantly higher than the NC group except DCAFH group.

Table 3.6: Serum creatinine, urea, AST and ALP levels in different animal groups at the end of the 7-week experimental period.

Serum parameters	NC	DBC	DCAFL	DCAFM	DCAFH	DM
Creatinine (µg/dL)	3.03±0.17	2.17±0.82	2.40±0.79	2.61±0.57	2.86±0.23	4.19±3.45
Urea (mg/dL)	41.67±6.92 ^a	66.57±3.49 ^b	46.33±8.59 ^a	52.57±11.46 ^{ab}	43.71±8.76 ^a	51.43±6.16 ^a
AST (U/L)	86.6±9.32	69.5±10.98	73.3±19.34	64.66±14.70	81.67±19.22	92.5±19.97
ALP (U/L)	104.29±16.82 ^a	655±128.26 ^d	303.4±68.80 ^c	269.60±91.92 ^c	141.75±24.88 ^{ab}	186.2±65.96 ^{ab}

The results are expressed as mean ± SD of 5-7 rats. ^{a-b}Different alphabets within a row are significantly different from each other (Tukey's-HSD multiple range post hoc test, P<0.05). AST: aspartate transaminase; ALP: alkaline phosphatase.

3.3.9. Thiobarbituric acid reactive substances (TBARS) assay

The data for the concentration of TBARS as the equivalent of MDA in the various tissues and serum samples of different animal groups are presented in **Figure 3.19**. Results show that there was a significant decrease in the MDA concentration ($p < 0.05$) in the DCAFH group in the brain, kidney, pancreas and serum compared to the DC group and the results were even lower than DM and NC groups in some samples. In the heart, there was no significant difference between the groups.

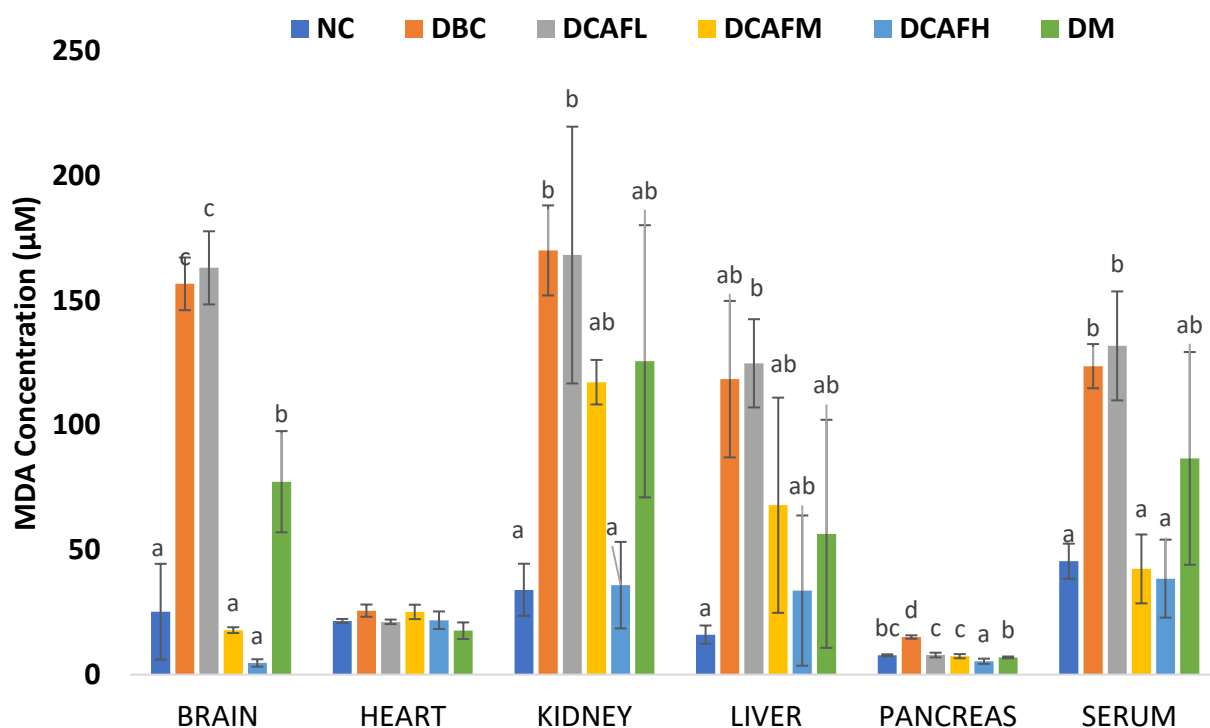


Figure 3.19: Malondialdehyde concentration in the serum and various organs of different animal groups at the end of the experimental period. The results are expressed as mean \pm SD of 5-7 rats. ^{a-d}Different alphabets above the bars for a given sample are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.3.10. Reduced glutathione (GSH) activity

Figure 3.20 highlights the results obtained for the GSH assay. The induction of diabetes (DC group) significantly decreased the GSH concentrations in the liver, pancreas and serum as compared to NC group. On the other hand, treatment with high dose of caffeine (DCAFH) significantly ($p < 0.05$) increased the GSH levels in the brain, liver, pancreas and serum samples compared to the DC group (**Fig. 3.20**).

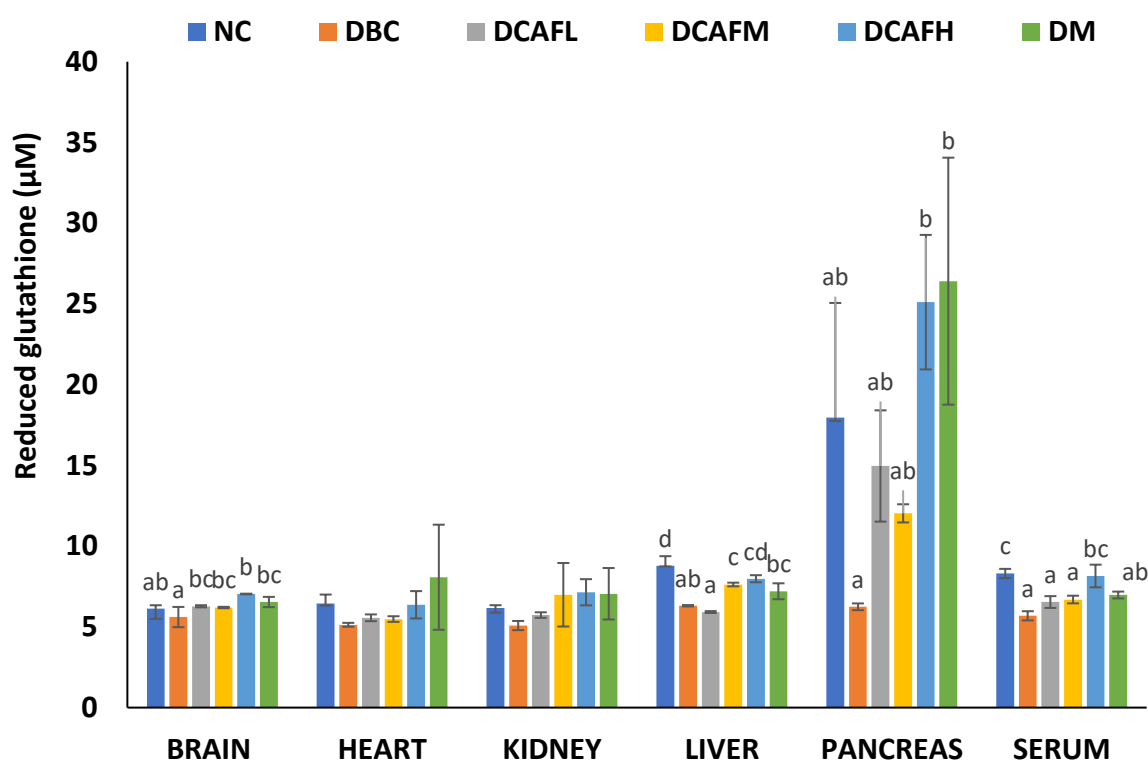


Figure 3.20: GSH concentration in different animal groups at the end of the experimental period. The results are expressed as mean \pm SD of 5-7 rats. ^{a-d}Different alphabets over the bars for a given sample are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$). GSH: reduced glutathione.

3.3.11. Catalase Activity

Figure 3.21 depicts the data obtained for the catalase assay. The NC, DCAFM, DCAFH and DM groups showed a significantly higher catalase activity compared to the DCB and DCAFL groups (Fig. 3.3.11).

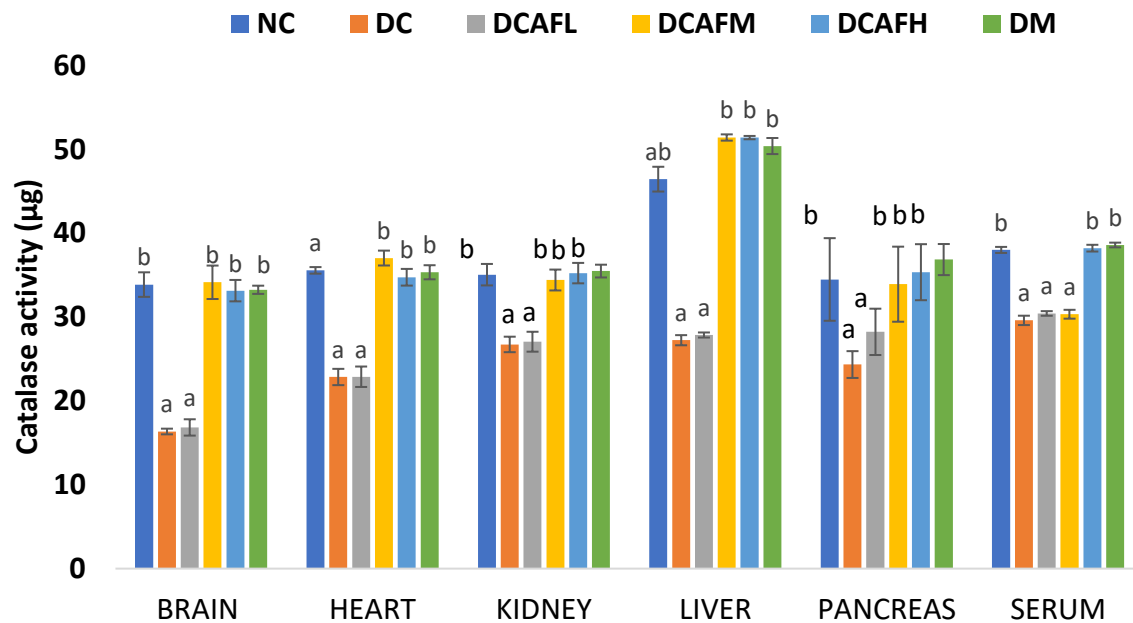


Figure 3.21: Catalase activity in different animal groups at the end of the experimental period. The results are expressed as mean \pm SD of 5-7 rats. ^{a-d}Different alphabets above the bars for a given sample are significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$).

3.3.12. Histopathological examination of the pancreatic tissue

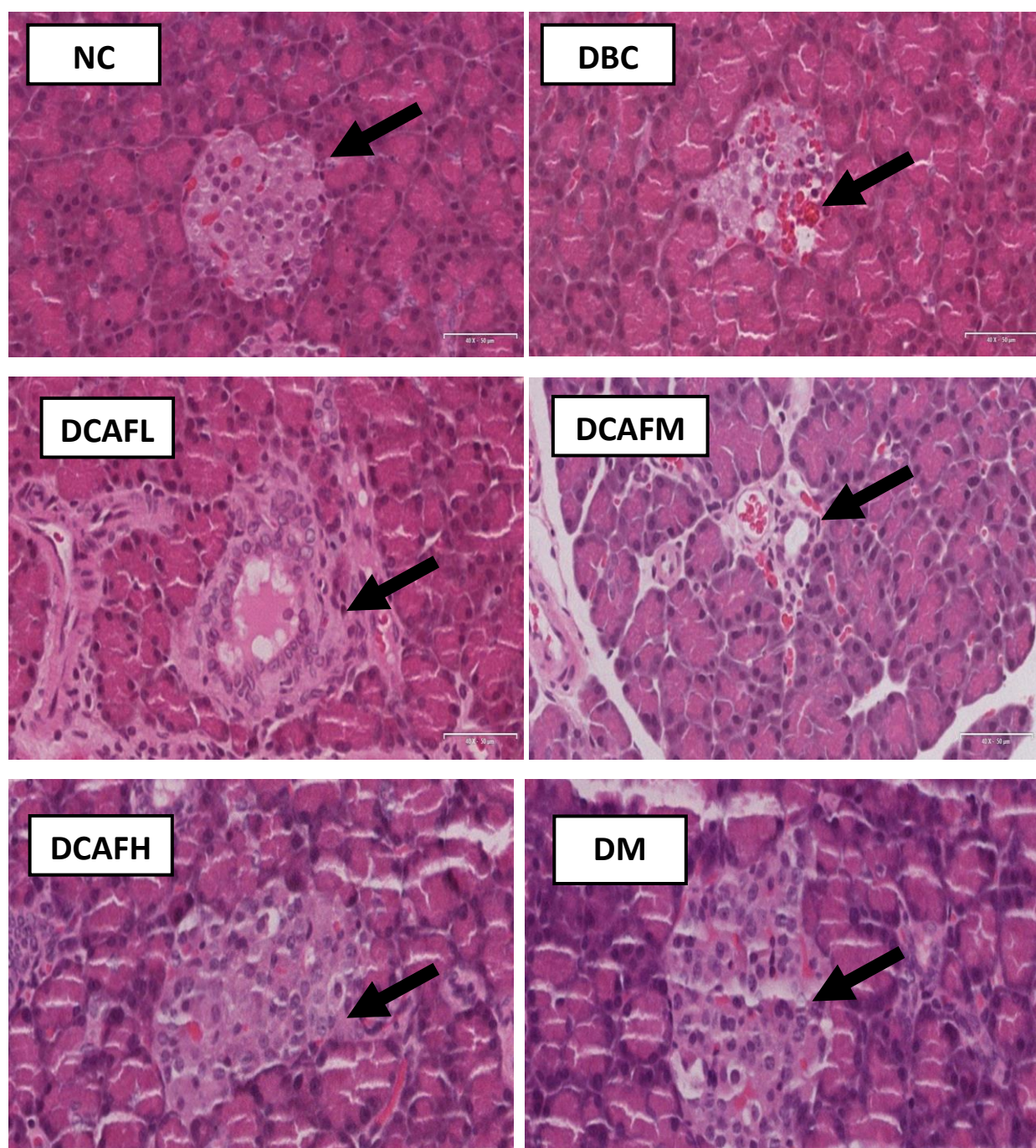


Figure 3.22: Histopathological examinations (40x) of the pancreatic islets of different animal groups at the end of the experimental period.

The slides for histopathological examination of the pancreatic tissues are presented in **Figure 3.22**. The induction of diabetes significantly damaged the pancreatic islets which was confirmed by the islets morphology of DBC group compared to the NC group. Although the treatment with the low (DCAFL) and medium (DCAFM) doses of caffeine could not recover the pancreatic beta-cell damage, a substantial recovery of pancreatic islets was observed in the DCAFH and DM groups compared to the DBC group (**Figure. 3.22**).

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. Discussion

Caffeine has been used for many years and is one of the most extensively consumed active food ingredient throughout the world (Andrews *et al.*, 2007). Caffeine has been broadly studied in a variety of areas regarding human health and performance (Smit and Rogers 2002). Research suggests that caffeine can aid in reducing symptoms associated with Parkinson's disease such as the deterioration of gross and small motor skills, and tremors (Trevitt *et al.*, 2009). Additionally, reports have shown that consumption of coffee or caffeine containing drinks are associated with the reduction of T2D related symptoms, promotes weight loss and acts as an antioxidant. (Bhupathiraju *et al.*, 2013; Whitehead and White, 2013). However, the fundamental mechanisms have not been identified. Elucidation of the causal mechanisms are needed before these findings can be put to therapeutic use. Therefore, the main objective of this study was to investigate the mechanisms behind the anti-diabetic effects of caffeine.

A common strategy for T2D management is through the usage of potent anti-oxidative agents. This method affords a therapeutic option for the amelioration of chronic vascular complications related to oxidative stress. Preliminary anti-oxidative tests were done to determine the antioxidant potential of caffeine at different doses using various models.

The model of scavenging the stable DPPH radical is a widely used to evaluate the antioxidant activity of pure compounds. DPPH is a free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Hence, DPPH is usually used as a substrate to evaluate the free radical scavenging activity of antioxidative agents *in vitro*. Using this model, caffeine demonstrated remarkable anti-radical activities (**Figure 3.1**) and a very low IC₅₀ value (**Table 3.1**). This suggests that this compound contains powerful free radical scavenging activities that could have the ability to ameliorate T2D-associated complications and indeed other oxidative stress associated metabolic disorders.

Antioxidants can be reductants that can inactivate oxidants and the reaction can be described as a redox reaction in which one reaction species is reduced at the expense of the oxidation of the other. Thus, the presence of antioxidant substances in the samples causes the conversion of the Fe³⁺/ferricyanide complex to the Fe²⁺ form which can be measured at 700 nm (Chung *et al.*, 2002). The reducing power of caffeine increased with increasing concentration, this suggests that the electron donating ability of the compound is concentration dependent. The higher reducing power of caffeine at the highest concentration used (**Figure 3.2**), suggest that the compound has dose dependent redox potentials that could act as reducing agents or hydrogen

donors (Kahkonen *et al.*, 1999). This is not surprising, since a recent study highlighted the antioxidant potential of moderate coffee consumption by human subjects (Belete and Bikila, 2015). Nitric oxide radicals are very unstable species that has been implicated in the pathology of cancer, T2D and numerous other ailments (Fukumura *et al.*, 2006). In this study, caffeine was able to inhibit the NO radical however in a non-dose dependant manner (**Figure 3.3**).

The hydroxyl radical is a highly potent free radical in living organisms and has been implicated as a highly damaging species in free radical pathology that can damage all life essential biomolecules (Kalaivani *et al.*, 2004). The hydroxyl radical scavenging activity is thus measured as the percentage of inhibition of these radicals generated in the Fenton's reaction mixture (Fe^{3+} /EDTA/ascorbate/ H_2O_2) by studying the competition between deoxyribose and caffeine (Hinnerburg *et al.*, 2006). Caffeine was found to be effective in hydroxyl radical inhibition (**Figure 3.4**). The pancreas has the lower antioxidant defence mechanisms compared to other organs when it is very vulnerable to hydroxyl radical (Quilliot *et al.*, 2005). The hydroxyl radical scavenging activity of caffeine also proves it pancreatic beta-cell protecting activity.

A commonly used and potent strategy for T2D management is the inhibition of α -glucosidase and α -amylase enzymes. This method affords a therapeutic option for the amelioration of hyperglycaemia. The action of carbohydrate hydrolyzing enzymes on carbohydrate-containing diet has a significant effect on postprandial blood glucose level and delaying the digestion of carbohydrate like starch and sucrose will translate into lower postprandial blood glucose (Dhital *et al.*, 2013; Reuser and Wisselaar, 1994). α -glucosidase and α -amylase inhibitors have gained much popularity as a class of hypoglycaemic agents, which decreases postprandial blood glucose via inhibition of the action of α -glucosidase and α -amylase enzymes (Patel *et al.*, 2012). Our findings revealed that caffeine did have the ability to inhibit α -amylase and α -glucosidase in a non-dose dependent manner, however, the results were significantly lower ($p < 0.05$) compared to acarbose in all concentrations used in this experiment (**Figure 3.5 and Figure 3.6**). Lower inhibitory activity of caffeine might be due to very low dose used in our study. However, our results are also supported by the results of a recent study (Alongi and Anese, 2018) where the efficiency of α -glucosidase inhibition by extracts obtained from roasted coffee beans was demonstrated.

Research has demonstrated the potential of natural products to counteract obesity. Natural products provide a vast pool of pancreatic lipase inhibitors. Caffeine supplementation has been recently considered as an effective means of weight management. It increases metabolism and

thus plays an effective role in weight management (Heckman *et al.*, 2010). It has been shown to increase energy expenditure in humans, and weight loss has reduced risk factors for diabetes in clinical trials (Ketzal *et al.*, 1995). Consequently, it seems possible that caffeine consumption may decrease diabetes risk by helping individuals control their body weight. This study demonstrated a dose-dependent anti-lipase activity of caffeine, the level of inhibitory activities was moderate might be due lower concentrations used in our study (**Figure 3.7**). Reports have shown that a wide variety of plant products such as polyphenols, flavonoids, and caffeine possess lipase inhibitory effects (Yun, 1010). An *in vivo* study also showed that caffeine stimulates fat breakdown (Mohammed *et al.*, 2014).

In vitro studies have shown that caffeine, even at millimolar concentrations, shows potent antioxidant power by preventing lipid peroxidation (Daniela *et al.*, 2017). These reports have suggested that the ability of caffeine to inhibit lipid peroxidation is similar to glutathione and significantly greater than that of ascorbic acid. Ferrous sulphate (FeSO_4) was used in this study to induce lipid peroxidation since the deleterious effects of iron has been earlier explained. Incubation of tissue homogenates with FeSO_4 has been shown to cause lipid peroxidation (Obboh *et al.*, 2007). In this study, the increased MDA level (**Figure 3.8**) shows an occurrence of Fe^{2+} -induced lipid peroxidation in the pancreatic tissues which shows successful induction of oxidative injury. The significantly lower ($p < 0.05$) MDA level in the pancreatic tissues indicates the reduction in lipid peroxidation by caffeine (**Figure 3.8**). Superoxide dismutase (SOD) and catalase (CAT) are vital antioxidant enzymes required for the protection against free radicals (Dudonne *et al.*, 2009). SOD which is produced primarily in the mitochondria (Genova *et al.*, 2001), catalyses the conversion of the superoxide radical to hydrogen peroxide, which is then converted to water and oxygen by catalase (Asmat *et al.*, 2016). A significant reduction ($p < 0.05$) in SOD and CAT were observed in the untreated control groups (**Figure 3.10 and Figure 3.11**). The reduction in these enzyme activities could be due to the derangement of the three isoenzymes of SOD by Fe^{2+} undergoing reduction Fe^{3+} during the Fenton reaction (Sanni *et al.*, 2018). The increased activity of SOD and CAT (**Figure 3.10 and Figure 3.11**) in the pancreatic tissue following the treatment of caffeine additionally reveals its antioxidative potency. The ability of these extracts to improve endogenous antioxidant status might be due to the presence of polyphenols and melanoidins in pure caffeine reported in a study (Metro *et al.* 2017)

The main dietary source of glucose for human is starchy foods and other comparable carbohydrates, and the degree of absorption of digestion of glucose from starch is an important

factor that plays a major role in the advancement of diabetes, obesity and related metabolic disorders (Dhital *et al.*, 2013). Glucose is rapidly absorbed in the intestine by a mechanism of facilitated diffusion, and results from previous *in vitro* studies have suggested the elevated capacity of glucose absorption across all segments of the small intestine (Fearson and Bird, 1968), with the proximal-to-mid small intestine (part of the duodenum and jejunum) having the highest absorption capacity (Lavin, 1976). In the present study, a 2-hour incubation of isolated rat jejunum in a glucose solution with caffeine showed that there was a significant and dose-dependent reduction ($p < 0.05$) in the glucose absorption capacity, when the higher inhibition of absorption was observed at concentrations of 120 and 240 $\mu\text{g/mL}$ (**Figure 3.12**). This is an indication of the possible inhibitory potentials of caffeine on intestinal glucose absorption, which is in line with its observed α -glucosidase and α -amylase enzymes inhibitory activity (**Figure 3.5 and 3.6**). The inhibitory activity of caffeine to these carbohydrate digesting enzymes might be partly involved in its mechanism behind the hypoglycaemic effect.

In T2D, glucose uptake by skeletal muscle is significantly reduced consequently, resulting in hyperglycaemia. Therefore, antidiabetic drugs have targeted to improve the glucose uptake as their mode of action. In the present study, the dose-dependent increase in muscle glucose uptake shown by caffeine, (**Figure 3.13**) additionally reveals its antidiabetic potentials. This further suggesting the potential of caffeine in increasing muscle glucose uptake, which in turn may decrease hyperglycaemia in T2D.

The promising *in vitro* antioxidant and anti-diabetic potentials of caffeine prompted the need to examine its antioxidative effects in a type 2 diabetes model of rats. The use of rat models in studying diabetes mellitus is well known and a useful method for investigating the protective effects of various natural and chemically originated agents on diabetes and its associated complications (Yilmaz *et al.*, 2014). A reliable T2D rat model must demonstrate the main pathological features of the disease which is insulin resistance as well as partial damage to the β -cells of the pancreas. The model used in this study was developed by Wilson and Islam (2012) and it successfully induced T2D by showing insulin resistance by feeding 10% fructose solution instead of drinking water followed by a low-dose STZ injection, which causes partial pancreatic β -cell damage.

Polydipsia, polyphagia and weight loss are some of the most common symptoms of diabetes which have been noticed in all diabetic groups in this experiment. These parameters are

generally dependant on energy expenditure, urinary excretion of glucose and catabolic processes among others. Although treatment with caffeine did not completely reverse the T2D induced polyphagia, polydipsia and weight loss, improvement of BW in the DCAFH group compared to the DC group (**Figure 3.15**) denotes the body weight ameliorative effect of caffeine.

Postprandial hyperglycaemia is a common pathogenesis of T2D which is induced by insulin resistance and partial pancreatic beta cell destruction (Wilson and Islam, 2012). Effective control of the blood glucose concentration is a vital step in ameliorating complications of diabetes and enhancing the quality of life in type 2 diabetic patients (Ross, 2004). In this study, oral treatment of the high dose of caffeine (DCAFH) resulted in a significant ($p < 0.05$) and consistent decrease in blood glucose levels (**Figure 3.16**) throughout the experimental period, signifying its potent anti-diabetic activity. Furthermore, the observed better glucose tolerance ability in the DCAFH group compared to the DBC group (**Figure 3.17**) also denotes its glucose tolerance improving activity.

T2D is associated with vast changes in serum lipid and lipoprotein profiles accompanied with a high risk of cardiovascular disease (Kumar *et al.*, 2011). Hyperlipidemia is an established impediment in diabetic subjects which is characterized by an increased serum cholesterol and triglycerides levels (Kumar *et al.*, 2011). This is primarily due to the uncontrolled actions of lipolytic hormones on the fat depots resulting from the impairment of insulin secretions in diabetic subjects. In this study, serum total and LDL-cholesterols were significantly increased ($p < 0.05$) and triglycerides were relatively increased after the induction of diabetes in rats (**Figure 3.18**). On the other hand, the significantly lower serum total cholesterol concentration in the DCAFL group than the untreated control group (**Figure 3.18**) denotes its hypolipidemic activity as well although no significant differences were observed for other groups or other lipid-related parameters. This observation is perhaps related to the stimulation of insulin secretion by the compound which could modulate the actions of the lipolytic hormones in fat reserves.

An important feature of experimentally induced diabetes is a decrease in the liver glycogen level which is caused by the modulation of glycogen synthase and glycogen phosphorylase enzymes activities. In our study, feeding high dose of caffeine (DCAFH) significantly replenished the liver glycogen level in diabetic animals compared to the DBC group (**Table**

3.4) might be due to the decreasing glycogen phosphorylase and/or increasing glycogen synthase activity caffeine. However, further studies are needed to ascertain this speculation.

The serum fructosamine level is a measure of early protein glycation which is formed by the covalent binding of glucose to serum proteins via a non-enzymatic glycation reaction (Huang *et al.*, 2011; Habibuddin *et al.*, 2008). It shows the overall level of blood glucose in last several weeks' time in a diabetic subject. In our study, the treatment with the high dose of caffeine (DCAFH) was found to ameliorate the T2D-induced increase in serum fructosamine level (**Table 3.5**) which again supports the hypoglycemic effects of caffeine. The level of serum insulin is usually reciprocal with the level of serum fructosamine in diabetic condition. The higher serum insulin levels observed in the high dose-treated (DCAFH) group suggest that caffeine has insulin secretagogue activity which has been further proven by calculated HOMA-IR and HOMA- β scores as discussed below.

A recent study reported that the patients with T2D have lower insulin secretory capacities with mild, but evident, insulin resistance (Urakami *et al.*, 2013). The HOMA- β and HOMA-IR scores are used to determine the insulin secreting ability of pancreatic beta-cells or the level of pancreatic β -cell function (HOMA- β) and the degree of insulin resistance (HOMA-IR), respectively. The significantly higher HOMA-IR scores and significantly lower HOMA- β scores in the DBC group compared to the NC group denotes the induction of insulin resistance and partial pancreatic β -cell dysfunction in our animal model. On the other hand, the significantly lower HOMA-IR scores and significantly higher HOMA- β scores in the DCAFH compared to the DBC group (**Table 3.5**) provide additional evidence that caffeine treatment at 40 mg/kg BW not only has insulin resistance ameliorative effects but also has pancreatic β -cell protecting ability in STZ-induced type 2 diabetic rats. These effects were also comparable with the standard anti-diabetic drug (metformin) used in this study (**Table 3.5**).

The serum levels of ALP but not AST were significantly increased in the untreated diabetic animals (**Table 3.6**) indicating impaired liver function, which is due to hepatocellular necrosis. Diabetic complications such as increased ketogenesis and gluconeogenesis may be due to the elevated aminotransferase activities. Thus, restoration of these enzymes towards near normal levels specifies decreased diabetic complications in the DCAFH and DM treated groups. Moreover, the DCAFH group reversed the T2D-induced increase in serum urea level (**Table 3.6**) which indicates the amelioration in renal impairment and related complications in diabetes. Reports have shown that creatinine is a biomarker for T2D and insulin resistance (Kumar *et al.*, 2012). Creatinine is a product of creatine metabolism which occurs in skeletal muscle, the major

centre for the action of insulin and subsequent glucose disposal. Muscle mass directly correlates to serum creatinine. Thus, the serum creatinine could be a reliable marker to assess insulin resistance (Kumar *et al.*, 2012). Thus, previous studies have demonstrated lower serum creatinine level in T2D human patients compared to normal subjects (Hjelmsaeth, 2010; Harita *et al.*, 2009) which collaborates with our findings (**Table 3.6**).

The anti-oxidative status of the serum and the several organs of the caffeine treated diabetic rats were examined to determine, whether or not, the anti-diabetic effects of the caffeine in various organs was obtained via improving anti-oxidative status or not. The severity of damage resulting in the brain, kidney, heart, liver and pancreatic tissues of the diabetes-induced groups were monitored using the TBARS, GSH and catalase assays. STZ causes an increase in ROS levels and free radicals, as seen in diabetic subjects, resulting in a huge role in tissue damage which is supported by several studies (Bergamini *et al.*, 2004; Forbes *et al.*, 2008; Evans *et al.*, 2003).

Apart from above studies, a number of other studies suggest that oxidative stress is involved in the etiology of numerous diabetic complications (King & Loeken, 2004; Yilmaz *et al.*, 2004; Kakker *et al.*, 1995; Rahman *et al.*, 2007). Oxidative stress in diabetic condition arises from the increased production of free radicals and/or diminished endogenous antioxidant activities (Piconi *et al.*, 2003). The level of lipid peroxidation (LPO) in physiological system is generally measured by the production of LPO end products as MDA equivalent. On the other hand, the determination of antioxidant capacity that supports the inhibition of LPO as a mode of action of natural antioxidants in diabetic patients (Solomon *et al.*, 2010) is measured by the levels of endogenous antioxidant such as reduced glutathione (GSH) and level of antioxidative enzymes activity such as catalase, super oxide dismutase, glutathione reductase and glutathione peroxidase. The results obtained from this study portrays a significant increase ($p < 0.05$) in MDA concentration in all organs and serum samples of the diabetic (DBC group) rats compared to normal rats (NC group) (**Figure 3.19**) confirms the induction of oxidative stress in diabetic condition. This increase is known to be evidence of excess free radical production which is characteristic of patients with T2DM (Bayir, 2005), owing to elevated oxidative stress. In this study, the administration of caffeine at a medium (20 mg/kg b.w) and high dose (40 mg/kg b.w.) showed a significant reduction ($p < 0.05$) in the MDA concentration in most organs (brain, kidney, liver, pancreas) and serum sample (**Figure 3.19**) indicating the oxidative stress reducing effect of caffeine in diabetic condition (Lykkesfeldt and Svendsen, 2007).

GSH is a non-enzymatic antioxidant that defends oxidative damage which is related to the pathogenesis of diabetes and diabetics tend to have very low concentrations of cellular GSH (Anthony *et al.*, 2009) and a declined NADPH levels (Maritim *et al.*, 2003). GSH has been reported to be one of the most vital scavengers of ROS (Zitka *et al.*, 2012). The level of GSH in the liver, pancreas and serum of DBC group was found to be significantly lower than the NC group (**Figure 3.20**). The data obtained from the GSH assay correlates with a study that found a decrease in GSH concentrations in the kidney, liver, heart, pancreas and serum of animals that were chemically induced with diabetes (Maritim *et al.*, 2003). However, the significantly higher GSH concentration in the brain, liver, pancreas and serum of DCAFH group compared to the DBC group (**Figure 3.20**) denotes the antioxidative status improving effects of caffeine diabetic condition.

Antioxidative enzymes such as catalase plays a crucial role in eradicating the harmful effects of ROS and free radicals towards tissue damages. The reduced activity of antioxidant enzymes that is observed in the various tissues of the untreated DBC group could make these specific organs vulnerable to oxidative stress (Kakkar *et al.*, 1995). Interestingly, there was a significant increase ($p < 0.05$) in the catalase activity of the tissues of animals administered with the medium and high dose of caffeine as well as the metformin group (**Figure 3.21**). The increased catalase activity in the organs and serums of the DCAFM and DCAFH groups suggests an increased anti-oxidative activity, indicating a protective effect against H_2O_2 superoxide radical. This effect of caffeine is also comparable with the results obtained for standard anti-diabetic drug (metformin) used in this study.

Interestingly, these results were further supported by the histopathological examinations of the pancreatic islets where DCAFH groups (**Figure 3.22**) had more as well as healthier pancreatic islets than the DBC group. The DCAFH group was found to have high *in vitro* anti-oxidative activity, suggesting that the protection of the pancreatic islets could be mediated through an anti-oxidative dependent mechanism because oxidative stress is an important biological phenomenon in the process of pancreatic β -cell damage in T2D. Kagami *et al.* reported that caffeine (100 mg/kg BW) pre-injection can successfully prevent the STZ-induced (65 mg/kg BW) pancreatic beta-cell damage in male Wistar rats (Kagami *et al.*, 2008). So, our results are also in line with this previous study.

4.2. Conclusion

In conclusion, our data suggest that caffeine displayed anti-oxidative activities and inhibited key enzymes linked to type 2 diabetes which could be exploited for the development of holistic therapeutic strategy for the control of postprandial blood glucose levels, T2D and chronic vascular complications. The results of this study demonstrate that caffeine can modulate T2D-induced oxidative stress in various organs and this mechanism plays a vital role in the overall anti-T2D effects of the compound. The various mechanisms of action are elicited through delaying small intestinal glucose absorption, increasing muscle glucose uptake, improving pancreatic β -cell function and stimulating insulin secretions rather than by increasing insulin sensitivity. Pancreatic histopathology showed that caffeine prevents T2D-induced pancreatic β -cell destruction and improves their functions. Considering all above, caffeine can be used an effective anti-diabetic supplement in ant-diabetic foods and food products. Further studies are still warranted in humans and experimental animals in order to determine the most effective and safer dose of caffeine for achieving its most anti-diabetic effects.

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